CYTOCHROME $P450_{scc}$ AND THE CHOLESTEROL SIDE CHAIN CLEAVAGE REACTION OF ADRENAL CORTEX MITOCHONDRIA

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THIS THESIS WAS COMPOSED BY MYSELF AND THE RESULTS DESCRIBED THEREIN ARE THE PRODUCT OF MY OWN WORK.

Signed .

Date April 1980

ABSTRACT OF THESIS

Cytochrome $P450_{scc}$, the terminal oxidase for the side chain cleavage of cholesterol to pregnenolone in bovine adrenal cortex mitochondria, was partially purified to a specific content of 10.2 nmoles cytochrome P450/mg protein with a recovery of 14%. The method involves iso-octane extraction of lyophilised mitochondria, ammonium sulphate fractionation in the presence of sodium cholate, butanol-acetone extraction, phosphate buffer extraction, a second ammonium sulphate fractionation, calcium phosphate gel adsorption and elution.

The cytochrome $P450_{scc}$ preparation has no detectable activity for the 11β-hydroxylation of deoxycorticosterone. Cholesterol: cytochrome $P450_{scc}$ molar ratios are reduced to 1:100 and the cytochrome has been isolated as a low spin haemoprotein. Adrenodoxin contamination in the preparation has been reduced to less than 1% on a molar basis to cytochrome $P450_{scc}$ and NADPH adrenodoxin reductase activity reduced to 4% of that in mitochondria. Sodium cholate, initially added to effect solubilisation, was reduced in the final preparation to a 3:1 molar ratio to cytochrome $P450_{scc}$. The molecular weight of cytochrome $P450_{scc}$, determined by gel filtration, was 200,000. The main bands of peroxidase activity observed using isoelectric focusing were at pH 5.3 and 5.4.

Further purification of cytochrome $P450_{scc}$ was limited by degradation of the cytochrome P450. A degradation product of cytochrome $P450_{scc}$ with a Soret absorption maximum at 412 nm which failed to bind carbon monoxide was observed. Ligands to cytochrome $P450_{scc}$, in particular 4-phenylimidazole and aminoglutethimide, were found to inhibit the degradation process.

The absorption maxima of low spin cholesterol depleted cytochrome $P450_{scc}$ are 416, 535 and 568 nm; addition of cholesterol results in the formation of a high spin cytochrome $P450_{scc}$-cholesterol complex and a decrease in absorbance at 416 and 568 nm, an increase in absorbance at 392 and 648 nm with isosbestic points at 406, 457, 533 and 588 nm.

The affinity of cholesterol binding to cytochrome $P450_{scc}$ has been determined from the spectral dissociation constant Ks. The spectral dissociation constant is related to the concentration of cytochrome $P450_{scc}$; the equilibrium constant for cholesterol association is $K_{equil} = 0.43 \pm 0.03 \times 10^{6}\ M^{-1}$ in 10 mM potassium phosphate buffer pH 7.4 at 20°C. Potassium chloride appears inhibitory to cholesterol induced low to high spin state change of cytochrome $P450_{scc}$.

The first order rate constant of cholesterol association with cytochrome $P450_{scc}$ is $k = 7.5 \times 10^{-4}\ sec^{-1}$ at 20°C and is decreased by the presence of potassium chloride and increased by calcium chloride. The activation energy for the first order rates of cholesterol association to cytochrome $P450_{scc}$ is 88 kJ $mole^{-1}$.

The first order rate constants of association of side chain monohydroxylated sterols (24-, 25- and 26-hydroxycholesterol) to cytochrome $P450_{scc}$ are similar to one another but differ from that of cholesterol, or the nuclear monohydroxylated sterols, 7α and 7β-hydroxycholesterol and 19-hydroxycholesterol. The maximal absorbance change induced and the initial rate of association however is dependent on minor variations in the position of the hydroxyl group within the sterol and to the optical isomers at the same site.

Cytochrome $P450_{scc}$-cholesterol is a one electron acceptor on titration with NADPH. Cytochrome $P450_{scc}$-cholesterol can be anaerobically reduced to the ferrous state which on oxygenation forms an oxygenated cytochrome $P450_{scc}$-cholesterol complex. This oxygenated complex autoxidises, in the absence of adrenodoxin, to ferric cytochrome $P450_{scc}$-cholesterol without oxidation of cholesterol. The decay of the oxygenated complex is first order, $k = 9.3 \times 10^{-3}$ sec^{-1} at $4^{o}C$. The rate of autoxidation is influenced by pH, ionic strength and the nature of bound sterol. The activation energy of autoxidation is 75 kJ $mole^{-1}$. Investigations into the fate of the electron equivalence as oxygenated cytochrome $P450_{scc}$-cholesterol complex autoxidises to the ferric haemoprotein were initiated and attempts were made to monitor hydrogen peroxide and superoxide anion generation.

Addition of equimolar amounts of adrenodoxin to cytochrome $P450_{scc}$-cholesterol, followed by stoicheiometric reduction under anaerobic conditions and then oxygenation allows single catalytic turnover cycles of cytochrome $P450_{scc}$ to be studied. This has allowed detection of intermediates in the conversion of cholesterol to pregnenolone and a precursor-product sequence of cholesterol-22-hydroxycholesterol-20,22-dihydroxycholesterol-pregnenolone to be established. Addition of oxidised adrenodoxin to oxygenated cytochrome $P450_{scc}$-cholesterol results in formation of 22-hydroxycholesterol; in the absence of added adrenodoxin no oxygenated product of cholesterol is formed. Multiple turnover cycles with deoxycorticosterone as substrate for cholesterol depleted cytochrome $P450_{scc}$ failed to show deoxycorticosterone conversion to corticosterone. Multiple turnover cycles with 7α-hydroxycholesterol as substrate for cholesterol depleted cytochrome $P450_{scc}$ confirmed that the side chain of this nuclear monohydroxylated cholesterol is cleaved to form 7α-hydroxypregnenolone.

ABBREVIATIONS

Cholesterol: 5-cholesten-3β-ol

7α-hydroxycholesterol: cholest-5-ene-3β, 7α-diol

7β-hydroxycholesterol: cholest-5-ene-3β, 7β-diol

20-hydroxycholesterol: cholest-5-ene-3β, 20-diol

22-hydroxycholesterol: cholest-5-ene-3β, 22-diol

24-hydroxycholesterol: cholest-5-ene-3β, 24-diol

25-hydroxycholesterol: cholest-5-ene-3β, 25-diol

26-hydroxycholesterol: cholest-5-ene-3β, 26-diol

20,22-dihydroxycholesterol: cholest-5-ene-3β, 20,22-triol

Pregnenolone: 3β-hydroxy-5-pregnen-20-ene

7α-hydroxypregnenolone: 3β, 7α-dihydroxy-5-pregnen-20-one

17α-hydroxypregnenolone: 3β, 17α-dihydroxy-5-pregnene-20-one

Progesterone: 4-pregnene-3,20-dione

17α-hydroxyprogesterone: 17α -hydroxy-4-pregnene-3,20-dione

deoxycorticosterone: 21-hydroxy-4-pregnene-3,20-dione

cortisol: 11β,17,21-trihydroxy-4-pregnene-3,20-dione

Sodium cholate: 3α, 7α, 12α-trihydroxycholanoic acid, sodium salt

Iso-octane: 2,2,4-trimethylpentane

ketamine: DL-2-(methylamine)-2-(2-chlorophenyl)cyclohexanone

aminoglutethimide: α-(p-aminophenyl)-α-ethylglutarimide

Steroids are numbered and rings lettered as in formula (1) IUPAC-IUB 1967 Revised Tentative Rules for Steroid Nomenclature (Eur. J. Biochem. 10, 1-19, 1969).

The configuration of substituents on sterols at C_{20} and C_{22} used in the text conforms to the IUPAC-IUB 1967 Revisted Tentative Rules for Steroid Nomenclature (Eur. J. Biochem. 10, 1-19, 1969). (20S)-20-hydroxycholesterol was previously known as 20α-hydroxycholesterol and (20R,22R)-20,22-dihydroxycholesterol was known at 20α,22R-dihydroxycholesterol.

ENZYMES

NADPH adrenodoxin reductase: NADPH:ferredoxin oxidoreductase E.C.1.6.7.1.

Horseradish peroxidase:donor:hydrogen-peroxide oxidoreductase E.C.1.11.1.7.

Catalase:hydrogen-peroxide:hydrogen-peroxide oxidoreductase E.C.1.11.1.6.

Glucose-6-phosphate dehydrogenase:D-glucose-6-phosphate:NADP 1-oxidoreductase E.C. 1.1.1.49.

CHAPTER 1

INTRODUCTION

The function of the adrenal cortex is the production of steroid hormones. The different classes of steroid hormone produced by the gland, based on their main physiological effect are glucocorticoids, mineralocorticoids and adrenal androgens and oestrogens.

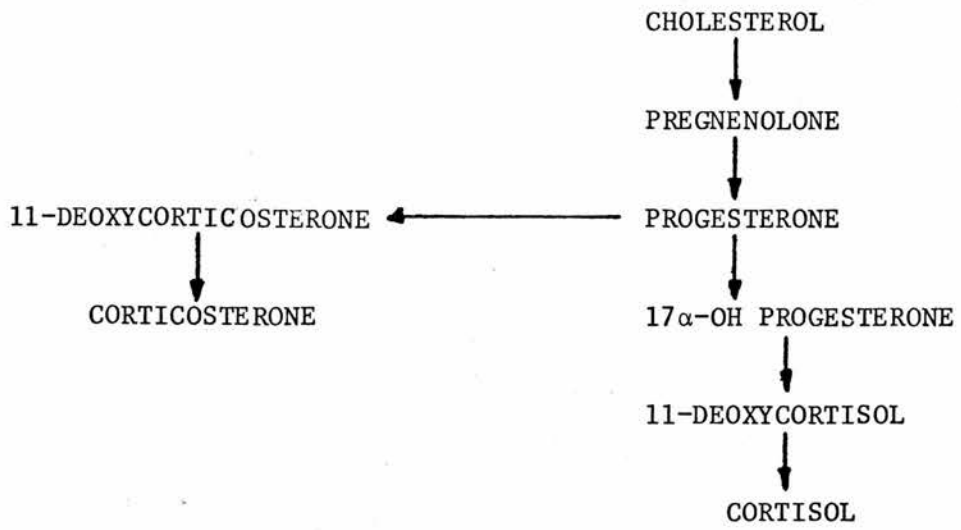
In man, the principal glucocorticoid is cortisol though some corticosterone is also produced. This contrasts with the rat where corticosterone is the primary glucocorticoid. The most important metabolic effect of the glucocorticoid is that on protein and carbohydrate metabolism causing the breakdown of peripheral protein in muscle, skin and connective tissue and a stimulation of gluconeogenic pathways in liver with increased synthesis of glycogen. The glucocorticoids have important effects on the regulation of fluid and electrolyte balance, however the more important effect is to increase glomerular filtration rate rather than facilitate sodium - potassium exchange on the distal nephron. Administration of cortisol leads to lymphopenia and atrophy of the spleen, thymus and lymph nodes. Cortisol also causes a reduction in the level of circulating eosinophils. Glucocorticoids have widespread effects on connective tissue with an inhibition of the formation and action of fibroblasts. Administration of glucocorticoids can prevent the manifestations of the inflammatory reaction perhaps by protecting the integrity of cellular and lysosomal membranes and preventing the release of substances responsible for the chain of events referred to as the inflammatory response.

The main mineralocorticoid is aldosterone and its principal physiological action is the stimulation of the active reabsorption of sodium by the distal nephron and facilitating the exchange of sodium for potassium or hydrogen ion at this site.

A major function of the foetal adrenal cortex is the secretion of dehydroepiandrosterone essential as a placental substrate for conversion to oestrogens. The deficiency states within the broad term congenital adrenal hyperplasia emphasises the critical role of balanced androgen and oestrogen secretion by the foetal adrenal in the normal development of female and male genitalia.

The corticoids have a 21 carbon structure based on 4-pregnene-3,20 dione with additional hydroxyl functions at 11β- and 21-positions in the case of corticosterone and at 11β-, 17α- and 21 in the case of cortisol.

In the 1950's, a pathway of steroid biosynthesis was established by Hayano et al. (1956) in which cortisol was shown to be synthesised solely by a route involving progesterone (Fig. 1.1). Following the discovery that dehydroepiandrosterone was a major end product of adrenal steroid biosynthesis, it was recognised that 17α-hydroxy-pregnenolone was an intermediate in its biosynthesis. This realisation was followed by proposals that 17α-hydroxypregnenolone was an intermediate in cortisol biosynthesis (Weliky and Engel (1961); Mulrow and Cohn (1961)). Another proposed intermediate is 17α,21-dihydroxypregnenolone, which was shown by Pasqualini et al. (1964) to be transformed to both cortisol and dehydroepiandrosterone.



ESTABLISHED PATHWAYS OF ADRENAL STEROID BIOSYNTHESIS

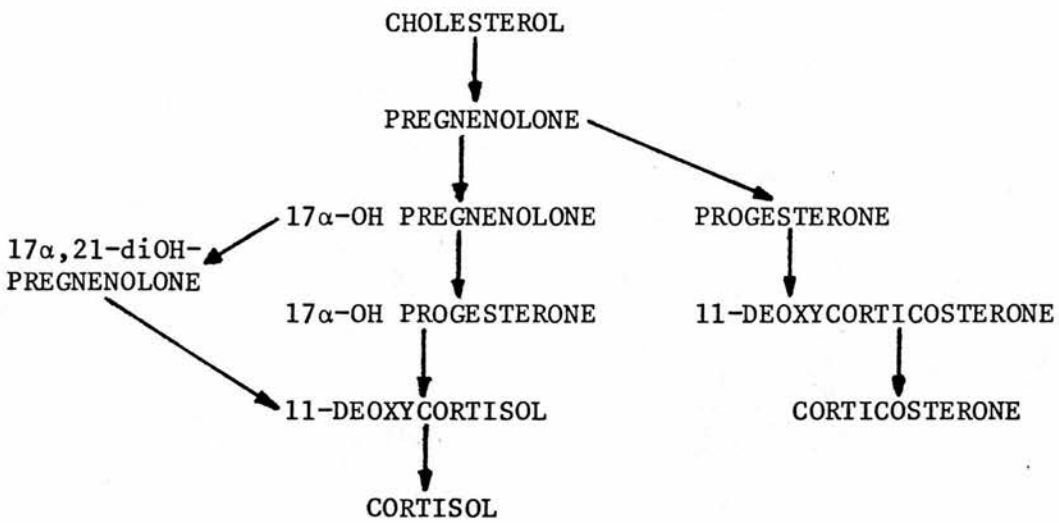


Fig. 1.1 ALTERNATIVE PATHWAYS OF ADRENAL STEROID BIOSYNTHESIS

The enzyme systems responsible for cholesterol side chain cleavage, steroid 11β-hydroxylation and steroid 18-hydroxylation are localised in the mitochondrial fraction of homogenates of adrenal cortex whereas the steroid 17α-hydroxylase and steroid 21-hydroxylase are present in the microsomal fraction. The steroid 3β-ol dehydrogenase has been considered to be an enzyme located exclusively in the endoplasmic reticulum but there is now evidence in adrenal and other steroid producing tissues that this enzyme has a bimodal distribution with activity present in the microsomal and mitochondrial fractions (Simpson and Mason (1976)).

Adrenal steroids can be synthesised from both acetate and cholesterol (Hechter and Pincus (1954)). However the role of cholesterol as an obligatory intermediate has been controversial. Hechter et al. (1953) perfused adrenal glands with radioactive cholesterol and acetate and after perfusion with acetate the specific radioactivity of cholesterol was much lower than that of the corticosteroid hormones. This suggested that either an alternative pathway from acetate to the steroid hormones existed or heterogeneity of the cholesterol pool was present. Werbin and Chaikoff (1961) observed that the specific radioactivities of urinary cortisol and adrenal cholesterol were equal after long term feeding of (4-^{14}C) cholesterol to guinea pigs. These results suggest that differences in specific activities of cholesterol and steroid hormones in short term experiments were due to differences in the rate of equilibration of different pools of cholesterol within the adrenal cortex.

The administration of ACTH causes a fall in the adrenal cholesterol concentration (Sayers et al. (1946)). The cholesterol in the mitochondria and microsomes is in the free form. However the bulk of the cholesterol in the gland is esterified and occurs in the lipid droplets which are

found in large numbers in the cytoplasm, Garren et al. (1971). The principal lipids in these droplets are cholesterol ester and phospholipid; the cholesterol appears to be selectively esterified to unsaturated fatty acids (Boyd and Trzeciak (1973); Goodman (1965)). Cholesterol ester hydrolase activity is increased by ACTH administration to rats with an increase in the pool size of free cholesterol available for the cholesterol side chain cleavage reaction (Boyd and Trzeciak (1973); Beckett and Boyd (1977)).

In most steroid hydroxylation reactions one atom of an oxygen molecule is inserted into the hydroxyl group of the product and the other oxygen atom is reduced to water. Mason (1957) gave the name 'mixed function oxidase' to such reactions which have the stoicheiometry: $NADPH + H^+ + R\text{-}H + O_2 \rightarrow NADP + R\text{-}OH + H_2O$. The requirement for molecular oxygen was demonstrated by Hayano et al. (1955) who showed the incorporation of $^{18}O_2$ during 11β-hydroxylation of deoxycorticosterone. The stoicheiometry of the equation was established by Cooper et al. (1963) for the 21-hydroxylation of 17α-hydroxyprogesterone by enzymes present in bovine adrenal microsomes.

Ryan and Engel (1957) showed that the 21-hydroxylation of 17α-hydroxyprogesterone was inhibited by carbon monoxide and the inhibition was reversed by light. Estabrook et al. (1963) on treating bovine adrenal cortex mitochondria with NADPH and carbon monoxide observed a pigment absorbing at 450 nm similar to that reported in liver microsomes by Klingenberg (1958) and Garfinkel (1958). This pigment was later shown to be a protoporphyrin haemoprotein of the b-type and named cytochrome P450 by Omura and Sato (1964). Estabrook (1963) demonstrated, by means of the photochemical action spectrum technique of Warburg (1949) that cytochrome P450 is the terminal oxidase for the steroid 21-hydroxylase. The presence of cytochrome P450 in adrenal cortex mitochondria was shown by Harding et al. (1964). Wilson et al.

(1965) demonstrated that carbon monoxide inhibited 11β-hydroxylation of deoxycorticosterone and Rosenthal and Cooper (1967) obtained the photochemical action spectrum. Similar results have been obtained for the cholesterol side chain cleavage reaction (Simpson and Boyd (1966); Wilson and Harding (1970)) and for the steroid 18-hydroxylase (Greengard et al. (1967)) in adrenal cortex mitochondria.

Omura et al. (1966) resolved the steroid 11β-hydroxylase system into three fractions, cytochrome P450, an FAD flavoprotein and an iron sulphur protein - adrenodoxin (Suzuki and Kimura (1965)); the steroid 11β-hydroxylase activity could be reconstituted when these three proteins were recombined. The cholesterol side chain cleavage system has been resolved into similar components (Simpson and Boyd (1967); Bryson and Sweat (1968)) as has the steroid 18-hydroxylase (Nakamura et al. (1969)). NADPH adrenodoxin reductase and adrenodoxin have been purified, crystallised and extensively characterised (Kimura et al. (1973)); Sugiyama et al. (1975)). These components appear to lack specificity as reconstituted with cytochrome P450 will support 11β-hydroxylation of deoxycorticosterone or the side chain cleavage of cholesterol. The concept of individual cytochrome P450 haemoproteins supporting the reactions of cholesterol side chain cleavage and 11β-hydroxylation of deoxycorticosterone has developed (Jefcoate et al. (1970); Ramseyer and Harding (1973); Shikita and Hall (1973)). The notations cytochrome $P450_{scc}$ and cytochrome P450 11β have been extensively used to distinguish the haemoprotein which acts as the terminal oxidase for cholesterol side chain cleavage from that associated with 11β-hydroxylation of deoxycorticosterone. The reaction sequence is as follows:

NADPH → Adrenodoxin Reductase → Adrenodoxin → Cytochrome $P450_{scc}$

Adrenodoxin → Cytochrome P450 11β

Cytochrome $P450_{CAM}$ is the terminal oxidase in Pseudomonas putida for the conversion of camphor to exo-5-hydroxycamphor (Katagiri et al. (1968)). This soluble cytochrome P450 has been purified and crystallised (Yu et al. (1974)) and has been used as a mechanistic model and guide for the purification of mammalian cytochrome P450.

Various steps of cyclic reduction and oxidation have been established for cytochrome $P450_{CAM}$ as it functions in oxygen activation and substrate hydroxylation (Estabrook et al. (1972); Gunsalus et al. (1972)). These reactions (Fig. 1, 2) may be summarised as follows:

a) The interaction of the low spin form of ferric cytochrome P450 with a substrate resulting in a characteristic optical change concomitant with the formation of a high spin state characteristic of the ferric cytochrome P450-substrate complex (Fe^{3+}-S).

b) The one electron reduction of the ferric cytochrome P450-substrate complex to ferrous cytochrome P450-substrate complex (Fe^{2+}-S).

c) The interaction of molecular oxygen with the ferrous cytochrome P450-substrate complex to form a ternary complex termed the oxygenated ferrous cytochrome P450-substrate complex.

d) The oxygenated form of the cytochrome P450-substrate complex undergoes a second one electron reduction to form an intermediate which as yet is ill defined. The mechanism of transfer of one atom of oxygen present in this complex to substrate with the resultant formation of water from the remaining atom of oxygen remains to be elucidated.

e) The formation of a complex of hydroxylated product with the cytochrome and the dissociation of this product to regenerate the low spin substrate depleted form of cytochrome P450, thus allowing for its participation in another reaction cycle.

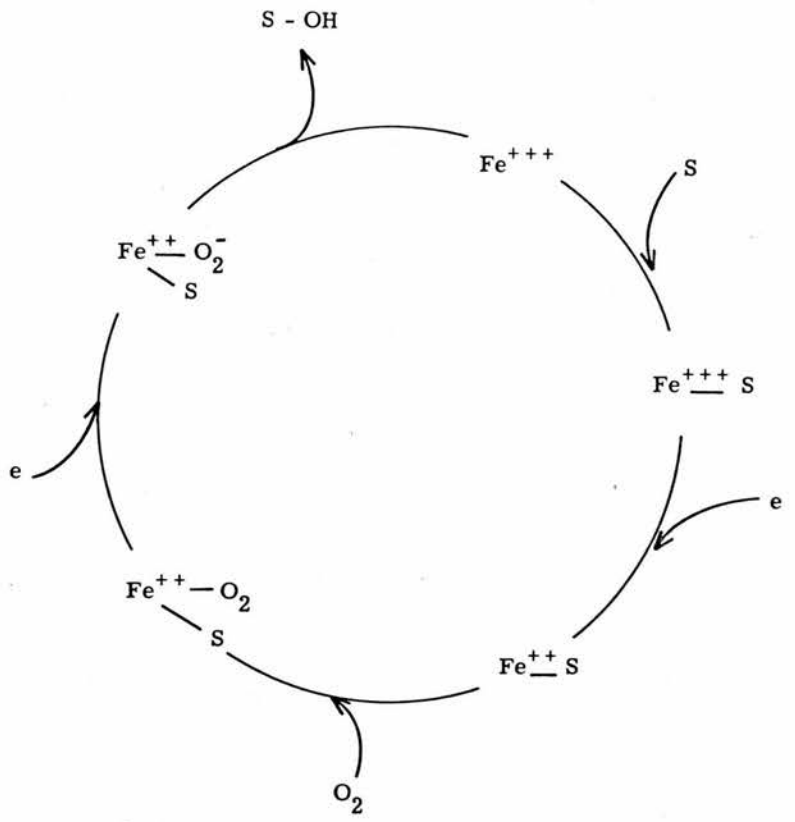


Fig. 1.2. THE CATALYTIC CYCLE OF CYTOCHROME $P450_{CAM}$ AS ESTABLISHED BY ESTABROOK et al. (1972) AND GUNSALUS et al. (1972) SHOWING THE STEPS OF CYCLIC REDUCTION AND OXIDATION OF IRON IN THE HAEMOPROTEIN AS IT FUNCTIONS IN OXYGEN ACTIVATION AND SUBSTRATE HYDROXYLATION.

The oxygenated complexes are shown in only one of the possible resonance forms. Abbreviations: S: substrate; S-OH: hydroxylated product; e: electron; Fe^{+++}-S: ferric-substrate complex; Fe^{++}-S: ferrous substrate complex; $Fe^{++}\!\!\begin{array}{l}O_2\\S\end{array}$: oxygenated ferrous-substrate complex and $Fe^{++}\!\!\begin{array}{l}O_2^-\\S\end{array}$: postulated complex formed on one electron reduction of the oxygenated ferrous-substrate form.

This cycle of enzymatic events has been established for cytochrome $P450_{CAM}$ and a similar reaction cycle has been assumed, but not established, for cytochrome $P450_{scc}$.

However certain features of cytochrome $P450_{scc}$ distinguish it from cytochrome $P450_{CAM}$. The conversion of cholesterol to pregnenolone is more complex than the single hydroxylation reaction catalysed by cytochrome $P450_{CAM}$ and may involve stable oxygenated derivatives of cholesterol. Suggested intermediates are (20S)-20-hydroxycholesterol (Shimizi et al. (1962); (22R)-22-hydroxycholesterol and (20R22R)-20,22-dihydroxycholesterol (Burstein et al. (1975); cholesterol-(20S)-20-hydroperoxide (Van Lier and Smith (1970)) and 20,22-epoxycholesterol (Kraaipoel et al. (1975).

The rate limiting step in steroidogenesis appears to lie between cholesterol and pregnenolone (Stone and Hechter (1954), current evidence suggests thatthe availability of cholesterol to the desmolase system is rate limiting (Mason et al. (1978b)). This is in contrast to cytochrome $P450_{CAM}$ where electron donation at the second reduction step is rate limiting to the overall reaction and not the availability or the binding of substrate (Gunsalus et al. (1972)).

Cytochrome $P450_{scc}$, unlike cytochrome $P450_{CAM}$, behaves as an integral protein of mitochondria (Singer and Nicholson (1972)) within the inner membrane (Yago and Ichii (1969)). The resolution of cytochrome $P450_{scc}$ from other membrane components, particularly the substrate cholesterol, is fundamental to the further understanding of this enzyme. The solubilisation and purification of cytochrome $P450_{scc}$ from bovine adrenal mitochondria has been attempted by a number of investigators (Ramseyer and Harding (1973); Shikita and Hall (1973); Horie and Watanabe (1975); Takemori et al. (1975) and Wang and Kimura (1976)). These preparations are of low specific

content in terms of nmoles cytochrome $P450_{scc}$/mg protein and are significantly contaminated by both cholesterol and detergent.

The topography of adrenal cortex mitochondria and the spatial relationships of cytochrome $P450_{scc}$, adrenodoxin and adrenodoxin reductase within the mitochondrial membrane have only recently become a focus of interest. At present no unified concept of structural relationships can be made and fundamental differences exist between authors in this area of research. However it is agreed that cytochrome $P450_{scc}$ behaves as a protein buried within the mitochondrial membrane and that its principal interactions within the membrane are hydrophobic in character (Churchill et al. (1978); Seybert et al. (1979)) requiring detergent action for solubilisation from the mitochondrial membrane. Reconstitution of cytochrome $P450_{scc}$ into phospholipid bilayers again suggests hydrophobic interactions rather than ionic and a dependence on the fatty acid composition of the phospholipid (Seybert et al. (1979)). Adrenodoxin forms a binary complex with cytochrome $P450_{scc}$ (Kido and Kimura (1979)) and this association requires cholesterol and a detergent or phospholipid. It is agreed that adrenodoxin acts as a peripheral protein in the membrane and that the association with cytochrome $P450_{scc}$ is partially ionic in character (Kido and Kimura (1979); Seybert et al. (1979)). Ternary complexes of cytochrome $P450_{scc}$, adrenodoxin and adrenodoxin reductase can be formed (Kimura et al. (1976)). The adrenodoxin-adrenodoxin reductase binary complex formation is dependent on ionic strength but is independent of cholesterol concentration (Kido and Kimura (1979)). Cytochrome $P450_{scc}$ and adrenodoxin reductase show no interaction and a binary complex of these two proteins has not been demonstrated (Kido and Kimura (1979)). These observations suggest that the

proteins of the cholesterol side chain cleavage system are located on the matrix surface of the inner mitochondrial membrane (Churchill et al. (1978)); that cytochrome $P450_{scc}$, adrenodoxin and adrenodoxin reductase are arranged linearly within the membrane and in the presence of high membrane cholesterol content the adrenodoxin-adrenodoxin reductase complex associates with cytochrome $P450_{scc}$ (Kido and Kimura (1979)). This scheme proposes a linearly mobile cytochrome $P450_{scc}$ within the membrane structure in contrast to the proposals of Seybert et al. (1979) where adrenodoxin provides a mobile shuttle between adrenodoxin reductase and cytochrome $P450_{scc}$. These schemes are not mutually exclusive and further research into the spatial relationships of the hydroxylase system within the mitochondrial membrane will be fundamental to the understanding of the control of steroid hydroxylation reactions.

The aims of this study were to gain a fuller knowledge of cytochrome $P450_{scc}$ from bovine adrenal cortex mitochondria and can be summarised:

a) the purification of cytochrome $P450_{scc}$ from bovine adrenal cortex mitochondria;

b) to establish a sequence of cyclic reduction and oxidation for cytochrome $P450_{scc}$, and

c) to identify intermediates in the conversion of cholesterol to pregnenolone and relate the intermediates to the catalytic cycle.

CHAPTER 2

MATERIALS AND METHODS

Chemicals

4-Phenylimidazole was supplied by Aldrich Chemical Co., (Gillingham). Ketamine hydrochloride was supplied by Parke, Davis and Co. (Pontypool). Sodium cholate, NADPH (tetrasodium salt, Type III), bovine serum albumin (Type F) and Antifoam A concentrate were obtained from Sigma Chemical Co., St. Louis. Aminoglutethimide was a gift from Ciba Laboratories (Horsham).

Potassium superoxide was supplied by Lancaster Synthesis Ltd. (Lancaster). Dicyclohexyl-18-crown-6 was kindly donated by Professor J.I.G. Cadogan. Potassium superoxide 50 mM was dissolved in a solution of 100 mM dicyclohexyl-18-crown-6 in dry dimethyl-sulphoxide, Valentine and Curtis (1975).

(20S)-20-hydroxycholesterol was synthesised by the method of Petrow and Stuart-Webb (1956). 24-hydroxycholesterol, 25-hydroxy-cholesterol and 26-hydroxycholesterol were synthesised by Dr. S.A.M. Ali (1968). (22R)-22-hydroxycholesterol was kindly given by Dr. Marcel Gut. (20R22R)-20,22-dihydroxycholesterol was a gift from Dr. J.I. Mason. 7α-hydroxycholesterol and 7β-hydroxycholesterol were synthesised by the photoxygenation of cholesterol, Schenck et al. (1959). 7α-hydroxypregnenolone was given by Professor Kirk, Medical Research Council Steroid Reference Collection. 19-hydroxycholesterol was available in this laboratory, previously synthesised in connection with other studies. Deoxycorticosterone was supplied by Sigma Chemical Co., St. Louis.

Calcium phosphate gel was purchased from British Drug Houses Ltd. Sephadex G-100 and Sepharose 4B were supplied by Pharmacia Fine Chemicals (Uppsala). DEAE-cellulose was supplied by Whatman, England.

(4-^{14}C) cholesterol 55 Ci/M and deoxy (1α2α(n)-^{3}H) corticosterone 42 Ci/mM were purchased from the Radiochemical Centre (Amersham).

All other chemicals were of Analar grade and supplied by British Drug House Ltd., (Poole) or Koch-Light (Colnbrook). Organic solvents were redistilled prior to use.

Enzymes

Horseradish peroxidase (Type II) and catalase (C-30) were purchased from Sigma Chemical Co., St. Louis. Glucose-6-phosphate dehydrogenase (Grade II) was obtained from Boehringer Mannheim GmbH, Mannheim.

Preparation of Adrenal Cortex Mitochondria

Bovine adrenal glands were obtained within 1 hr of slaughter and placed on ice. All subsequent operations were carried out between 0° and 5°C. The glands were trimmed of fat, cut in half about their longitudinal axis and the medulla removed by dissection. The cortex was scraped from the gland capsule and homogenised in 10 volumes of 10 mM potassium phosphate buffer pH 7.4 containing 0.25 M sucrose and 1 mM EDTA using a teflon pestle in a glass homogeniser. The homogenate was centrifuged at 650 g for 10 min producing pellet and supernatant fractions. The supernatant was centrifuged again at 650 g for 10 min to remove debris carried over during the first decantation and the pellet was rehomogenised and recentrifuged as above. The combined supernatants were centrifuged at 8,500 g for 20 min and the mitochondrial pellet formed resuspended by a single stroke of a loose fitting teflon-glass homogeniser in 20 volumes of 0.154 M potassium chloride containing 1 mM EDTA and centrifuged at 10,000 g for 20 min. The washed mitochondrial pellet was resuspended in a minimum volume of distilled water and lyophilised. Lyophilisation resulted in minimal loss of specific content of cytochrome P450 but with the advantage that the organelle structure was disrupted such

that on subsequent extraction with phosphate buffer, soluble proteins particularly adrenodoxin and NADPH adrenodoxin reductase were recovered in the supernatant fraction on centrifugation.

Preparation of Adrendoxin

The method usedfor preparation of adrenodoxin is a modification of the technique described by Orme-Johnson and Beinert (1969). Adrenal glands were trimmed of fat, cut in half about the longitudenal axis; the medulla removed by dissection and the cortices frozen at $-20^{o}C$ one layer deep in aluminium foil. The cortices were stored frozen for at least 2 weeks and then 150 gms thawed by placing in a litre of 0.25 M sucrose, 10 mM Tris hydrochloride pH 7.4 with 1 mM EDTA at $4^{o}C$. The tissue was stirred for 4 hrs and then the sucrose replaced with 0.15 M potassium chloride, 10 mM Tris hydrochloride pH 7.4 with 1 mM EDTA. The cortices were left to wash overnight and then removed and rinsed quickly with distilled water to remove as much lipid and blood as possible. The washed tissue was then homogenised in a Waring blender for 4 mins at maximal speed in a litre of 0.5 M potassium chloride, 10 mM Tris hydrochloride pH 7.4 and 1 mM EDTA. Homogenisation was effected for 30 sec periods with 30 sec intervals when the blender was place in ice. The homogenate was filtered through six layers of cheese-cloth to remove larger particles and the filtrate sonicated by an Ultrasonics Rapidis 150 with the specifications: amperage 3, frequency 20 kc on maximal settings. The homogenate was sonicated for 30 sec periods with 30 sec intervals for 10 mins. The homogenate was filtered again through six layers of cheese-cloth to remove collected lipid and the entire volume was sonicated again. The homogenate was centrifuged at 105,000 g for 90 mins and the floating lipid layer and the pellet discarded. To the infranatant was added about 150 mls of a thick slurry of DEAE-

cellulose, equilibrated with 10 mM potassium phosphate buffer pH 7.4. The mixture was allowed to stir overnight and then centrifuged at 5,000 for 20 mins. The pellet of DEAE-cellulose was collected in as small a volume as possible and stored frozen at -20°C. When required, the DEAE-cellulose was defrosted and washed twice with one litre aliquots of 10 mM potassium phosphate buffer pH 7.4 containing 0.1 M potassium chloride. The DEAE-cellulose was recovered by centrifugation at 5,000 g for 20 mins and the supernatant discarded. The washed DEAE-cellulose was stirred for 1 hr with 100 mls of 10 mM potassium phosphate pH 7.4 containing 0.5 M potassium chloride and the supernatant on centrifugation retained. This process was repeated twice. The combined supernatants were diluted five fold with 10 mM potassium phosphate buffer pH 7.4 and centrifuged at 20,000 g for 30 mins to remove turbidity. The supernatant was passed onto a DEAE-cellulose column 2.5 cms by 15 cms pre-equilibrated with 10 mM potassium phosphate buffer pH 7.4. The adrenodoxin adsorbed as a narrow band at the top of the column which was then washed with 500 mls of 10 mM potassium phosphate buffer pH 7.4 containing 0.17 M potassium chloride. Adrenodoxin was eluted with a minimal volume of 10 mM potassium phosphate pH 7.4 containing 0.33 M potassium chloride and the eluate chromatographed on a column of Sephadex G-100, 60 cms by 2.5 cms, equilibrated with 5 mM Tris hydrochloride pH 8.0 and containing 0.1 M potassium chloride. The spectral properties of adrenodoxin, showing absorbance maxima at 415 nm and 455 nm were used for identification of this protein in the eluate. Fractions containing adrenodoxin with ratios of absorbance 415 nm/absorbance 280 nm greater than 0.6 were pooled and transferred to a small DEAE-cellulose column 5 cms by 2 cms equilibrated with 10 mM potassium phosphate buffer pH 7.4. The adrenodoxin was eluted with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M potassium chloride. Selected fractions were dialysed

against 10 mM potassium phosphate buffer pH 7.4 before storage in small aliquots at -20°C.

Preparation of 7α-Hydroxypregnenolone

The 7α-hydroxypregnenolone was prepared by the photoxygenation method of Schenck et al. (1959). Oxygen was bubbled through a solution of 100 mgs pregnenolone, 5 mgs haematoporphyrin in 10 mls chloroform and irradiated with a fluorescent light source for 12 hrs. The pregnenolone hydroperoxides formed were reduced to the corresponding hydroxypregnenolones by the addition of 500 mgs potassium iodide in four drops of 10% acetic acid in ether:ethanol::1:5 by volume and by gently warming for 45 mins. Excess iodine was removed with sodium thiosulphite. The organic solvent was evaporated and the residue chromatographed on preparative thin layer silica gel plates developed in the solvent system benzene:ethyl acetate::7:13 by volume. The 7α-hydroxycholesterol Rf 0.3 was eluted from the silica gel with chloroform:methanol::2:1 by volume and crystallised from methanol with a yield of 15 mgs. The synthesised 7α-hydroxypregnenolone behaved on thin layer chromatography and had a mass spectrum identical to authentic 7α-hydroxypregnenolone from the Medical Research Council Steroid Reference Collection.

Preparation of (4-^{14}C) 7α-Hydroxycholesterol

The (4-^{14}C) cholesterol, specific activity 55 μCi/mole, was purified immediately before use by thin layer chromatography on silica gel developed in diisopropyl ether:petroleum spirit:acetic acid:::70:30:2 by volume. The region corresponding to standard cholesterol was eluted from the thin layer plate using redistilled acetone.

The (4-^{14}C) cholesterol, 1.0 μCi in 50 μl acetone, was added to an incubation medium of 7 mls containing 100 mM potassium phosphate buffer pH 7.4, 10 mM β-mercaptoethylamine, 5 μmole NADP, 50 μmole glucose-

6-phosphate, 1 unit glucose-6-phosphate dehydrogenase and approximately 10 mgs liver microsomal protein equivalent to 1 gm wet weight rat liver (Mitton et al. (1971)). After incubation at 37°C for 60 mins the reaction was stopped with 10 mls methanol and a lipid extraction performed with 15 mls chloroform followed by 10 mls boiling ethyl acetate. The sterols in the combined extracts were separated by thin layer chromatography using silica gel H in the solvent system benzene: ethyl acetate::7:13 by volume. The area of radioactivity corresponding to standard 7α-hydroxycholesterol was eluted from the thin layer plate using redistilled acetone.

The 7α-hydroxycholesterol concentrations were determined as free sterols by gas liquid chromatography and the (4-^{14}C) 7α-hydroxycholesterol chromatographed as a single peak. The specific activity of the prepared (4-^{14}C) 7α-hydroxycholesterol was 0.42 μCi/mole. The mass spectrum of (4-^{14}C) 7α-hydroxycholesterol was identical to that prepared by photoxygenation of cholesterol and reduction.

Cytochrome P450 Assay

Cytochrome P450 concentrations were determined from the reduced carbon monoxide difference spectrum (Omura and Sato (1964)) and extinction coefficient of 95 $mM^{-1}cm^{-1}$ (Gunsalus et al. (1972) for the 446 minus 490 nm absorption difference. An extinction coefficient of 106 $mM^{-1}cm^{-1}$ was used for absolute spectra of the reduced carbon monoxide complex of cytochrome at 446 nm (Gunsalus et al. (1972).

Spectral dissociation constants and type of spectral change on ligand binding as defined by Schenkman et al. (1967). An extinction coefficient of 110 $mM^{-1}cm^{-1}$ was used for difference spectra of substrate induced low to high spin state changes and an extinction coefficient 51 $mM^{-1}cm^{-1}$ for the decrease in absorbance in the absolute spectrum of the low spin species (Peterson (1971)).

Adrenodoxin Assay

Electron paramagnetic resonance spectra were recorded using a Varian E4 operated at -172°C. Adrenodoxin was quantitated from the height of the signal at g = 1.94 by comparison with a purified preparation (Estabrook et al. (1973)). The generic name iron sulphur protein is used where there is EPR evidence of iron sulphur protein of respiratory chain origin contaminating the adrenodoxin EPR spectrum.

NADPH Adrenodoxin Reductase Assay

The method was based on that of Omura et al. (1966) and results expressed in nmoles 2,6-dichlorophenolindophenol reduced/min/nmole cytochrome P450.

Protein Determination

Protein was assayed according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Cholate Determinations

Cholate levels were determined by radioimmunoassay (Beckett et al. (1978)).

Catalase Assay

Catalase activity was determined spectroscopically and activity expressed in international units (Aebi (1974)).

Phospholipid Determinations

Phosphorus was determined by the method of McClare (1971). Phospholipids and protein in aqueous solution were precipitated by addition of a 10% solution of trichloracetic acid and incubated at 4°C for 30 mins. The precipitate was extracted with chloroform:methanol:: 2:1 by volume, the solvent extract was taken to dryness by a stream of nitrogen and the phosphorus liberated from the resultant film by 0.5 ml perchloric acid. The colorimetric reagent contained 8% perchloric acid, 1% ammonium molybdate and 0.2% ascorbic acid, 9.5 mls of this reagent

was added to each tube and incubated at 50°C for 40 mins. The absorbance at 825 nm was recorded against a reagent blank and triphenyl phosphine was used as phosphorus standard. The absorbance change was linear in the range 1-7 µg phosphorus. The mass of phosphorus was multiplied by 25 to convert it to the mass of phospholipid. The average molecular weight of a phospholipid molecule was taken as 760.

Cholesterol Determinations

Cholesterol concentrations, as the free sterol, were determined by gas liquid chromatography on a 180 cm x 0.6 cm column of 0.5% SE30 on Gas-Chrom Q support using a Pye series 104 gas chromatograph, The conditions were: column temperature 240°C; attenuation 10 x 10^2; nitrogen flow rate 30 ml/min; hydrogen flow rate 30 ml/min and air flow 10 ml/min. The retention time of cholesterol was 10 min, pregnenolone acetate was used as an internal standard.

Cholesterol was extracted from cytochrome $P450_{scc}$ with methanol: chloroform:water:::1:2:3 by volume and the organic and aqueous phases separated by centrifugation. The aqueous phase was discarded and the organic phase carefully decanted into a boiling tube leaving the protein which had formed an interface with the organic and aqueous phases. The protein was re-extracted in a stoppered tube with 20 ml of hot ethyl acetate and the protein removed by centrifugation. The ethyl acetate was decanted and the protein re-extracted with the same solvent. The combined organic solvent extracts were taken to dryness in a warm bath and evaporated by a stream of air. Each residue was dissolved in 0.2 ml of chloroform.

The residue dissolved in chloroform was transferred to a thin layer plate (28 cm x 6.5 cm) coated with Silica gel H (Merck) and the thin layer plates developed in the solvent system; diisopropyl ether:petroleum spirit:acetic acid:::70:30:2 by volume. The area of

silica gel corresponding to cholesterol was extracted with an excess volume of chloroform:methanol::2:1 by volume which was reduced to dryness with nitrogen gas and made up to a standard volume in acetone. The efficiency of extraction of cholesterol was monitored by addition of 0.1 μCi (4-^{14}C) cholesterol to cytochrome P450 and its recovery after extraction and chromatography determined. The recoveries were in the range 95-98% of radioactivity added.

Estimation of Hydrogen Peroxide Determinations

The estimation of hydrogen peroxide was based on a method devised by Andreae (1955) in which the extent of loss of the fluorescence of a peroxidase substrate scopoletin (6-methyl-7-hydroxy-1:2 benzopyrone) is proportional to hydrogen peroxide concentration with a stoicheiometry of one mole of hydrogen peroxide required for the oxidation of one mole of scopoletin. This method was further refined by Perschke and Broda (1961) and detection of hydrogen peroxide in the range 10^{-12}M was observed. Further modification was made by Hildebrandt and Roots (1975) to allow detection of hydrogen peroxide based on scopoletin horseradish peroxidase added directly to a mitochondrial incubation and following the fluorescence decay with time.

A calibration of the decrease in the fluorescence of scopoletin in the presence of known amounts of hydrogen peroxide was established. Scopoletin (Sigma), stock solution 2.5 mM in 10 mM potassium phosphate buffer pH 7.4 was added to a concentration of 2.5 μM in a cuvette containing 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA. Horseradish peroxidase (Sigma type II), stock solution 20 mgs/ml in a 10 mM potassium phosphate buffer pH 7.4, was added to the cuvette to give a final concentration of 40 μg/ml. Hydrogen peroxide solutions were prepared by diluting Perhydrol with oxygen free 10 mM potassium phosphate buffer pH 7.4. The hydrogen peroxide concentration was standardised

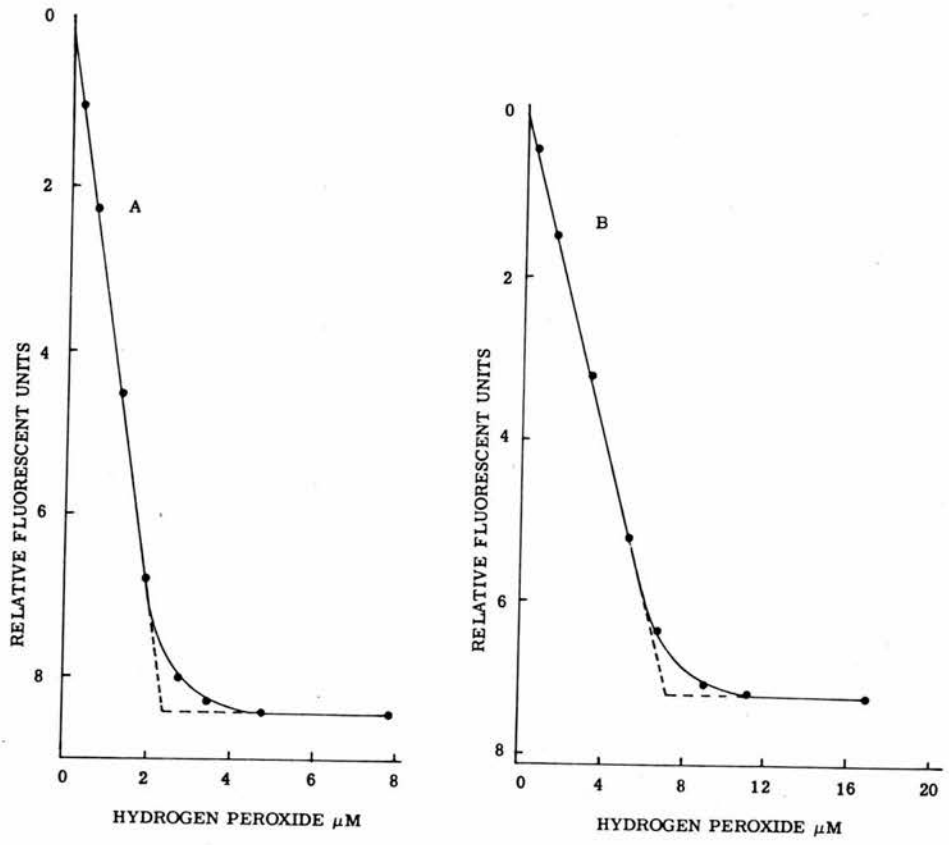


Fig. 2.1. THE RELATIONSHIP BETWEEN DECREASE IN RELATIVE FLUORESCENT UNITS OF SCOPOLETIN VERSUS HYDROGEN PEROXIDE CONCENTRATION

Scopoletin was added at a concentration of 2.5 μM in a cuvette containing 10 mM potassium phosphate buffer, 1 mM EDTA. Horseradish peroxidase was added to the cuvette and the decrease in fluorescence after each addition noted, showing a stoicheiometry of one mole of hydrogen peroxide required for the oxidation of one mole of scopoletin (Fig. A). However when the experiment was repeated under the same conditions with cytochrome $P450_{scc}$ added to 15 μM, some quenching of fluorescence was noted and the molar ratio of hydrogen peroxide added to scopoletin oxidation was 2.8:1 (Fig. B).

spectrophotometrically at 240 nm, by using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ and further dilution to give a stock solution of 7.6 mM in oxygen free 10 mM potassium phosphate buffer pH 7.4. Fluorescence measurements were made on a Perkin Elmer spectrofluorimeter Model MPF2A with excitation wavelength 350 nm; emission wavelength 450 nm; reference sensitivity on direct and sample sensitivity setting 2. The decrease in fluorescence of scopoletin on addition of hydrogen peroxide is shown in Fig. 2.1. The experiment was repeated under the same conditions with cytochrome $P450_{scc}$ added to 15 μM.

Gas Liquid Chromatography of Sterols

Concentrations of cholesterol, 20α hydroxycholesterol and 22R-hydroxycholesterol were determined as the free sterols by gas liquid chromatography on a 180 cm x 0.6 cm column of 0.5% SE30 on Gas-Chrom Q support using a Pye series 104 gas chromatograph. The conditions were: column temperature 240°C; heater mark 2; nitrogen flow rate 30 ml/min; hydrogen flow rate 30 ml/min and air flow 10 ml/min. The retention times of cholesterol, 20α hydroxycholesterol and 22R-hydroxycholesterol were 10, 12.5 and 18.5 min respectively. Cholesterol was used as internal standard for 22R-hydroxycholesterol determinations and pregnenolone acetate as internal standard for cholesterol and 20α hydroxycholesterol. The calibration curves for cholesterol and 22-hydroxycholesterol are given in Figs. 2.2 and 2.3.

Concentrations of 7α-hydroxycholesterol were determined by gas liquid chromatography with the above conditions, (Craig (1978)). The retention time was 6 mins and pregnenolone acetate was used as internal standard.

Sterols were recovered from biological material by solvent and thin layer chromatography with appropriate standards. The areas of silica

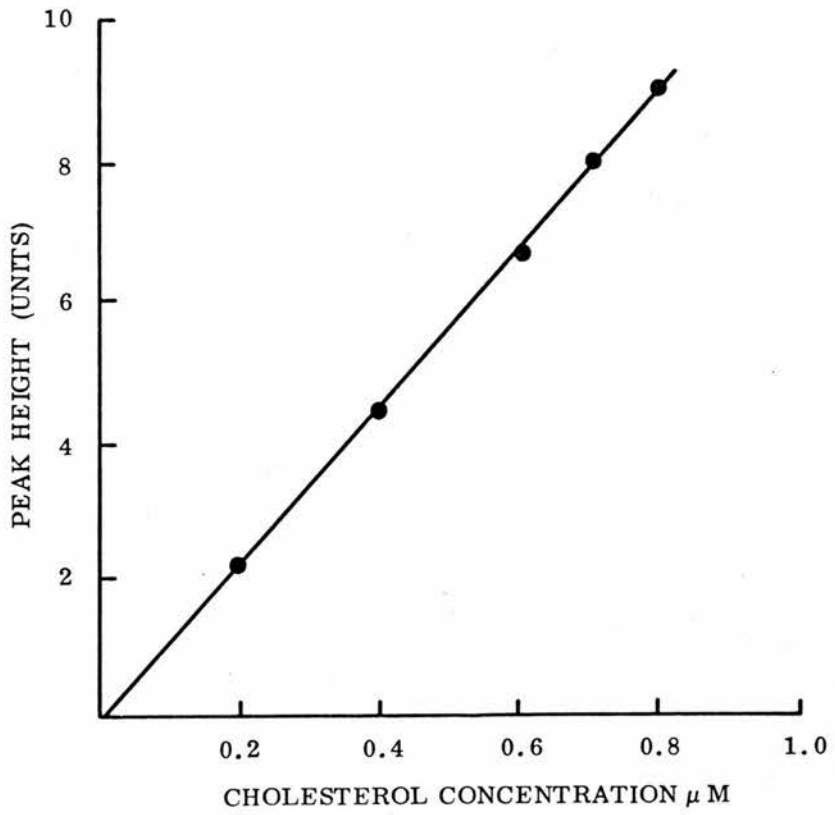


Fig. 2.2. CALIBRATION CURVE OF MASS OF CHOLESTEROL VERSUS PEAK HEIGHT OF DEFLECTION DETERMINED BY GAS LIQUID CHROMATOGRAPHY

Peak height is given in arbitrary units and at this attenuation, fullscale deflection is 10 units. The column was 180 cms x 0.6 cms of 0.5% SE30 on Gas-Chrom Q support and using a Pye 104 series chromatograph. The conditions were: column temperature 240^{o}C, heater mark 2, attenuation 50 x 10^{2}, nitrogen flow rate 30 ml/min, hydrogen flow rate 30 ml/min and air flow 10 ml/min. The stock solution of cholesterol was 0.2 mgs/ml dissolved in redistilled acetone.

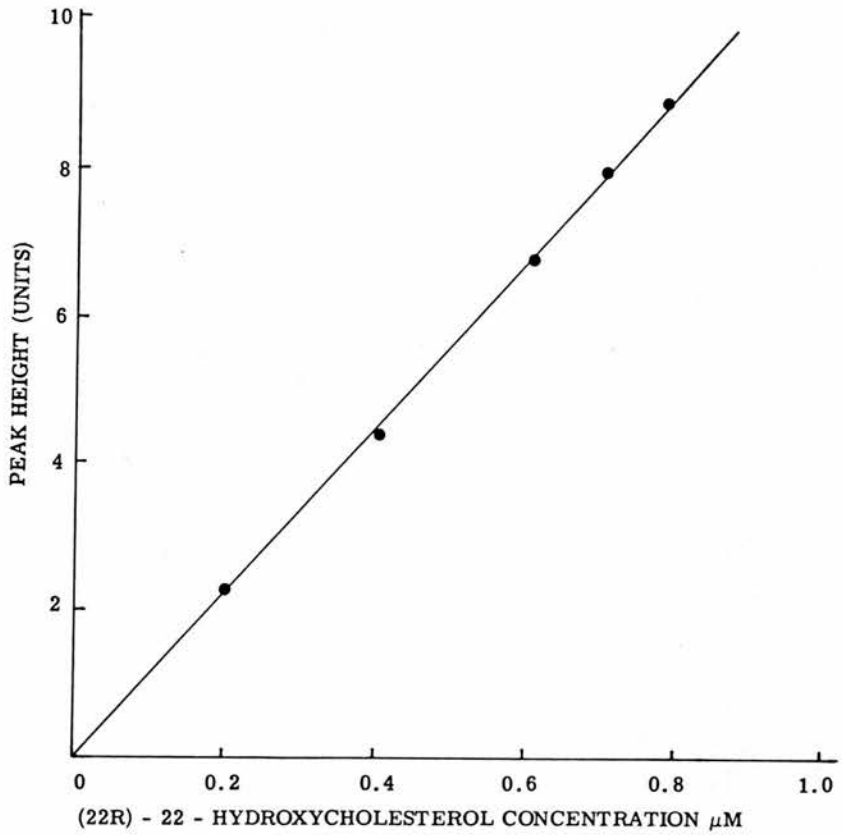


Fig. 2.3. CALIBRATION CURVE OF MASS OF 22R-HYDROXYCHOLESTEROL VERSUS PEAK HEIGHT OF DEFLECTION DETERMINED BY GAS LIQUID CHROMATOGRAPHY

Peak height is given in arbitrary units and at this attenuation, full scale deflection is 10 units. The column was 180 x 0.6 cm of 0.5% SE30 on Gas-Chrom Q support and using a Pye 104 series chromatograph. The conditions were: column temperature 240°C, heater mark 2, attenuation 10 x 10^2, nitrogen flow 30 ml/min, hydrogen flow rate 30 ml/min and air flow 10 ml/min. The stock solution of 22R-hydroxycholesterol was 0.2 mgs/ml dissolved in redistilled acetone.

gel corresponding to a sterol standard were extracted with chloroform: methanol, 2:1 by vol, reduced to dryness in a vial with nitrogen gas and made up to a standard volume in acetone. The efficiency of extraction of sterols was monitored by addition of 0.1 μCi (4-^{14}C) cholesterol to the biological material and recovery after extraction and chromatography determined. Recoveries were in the range 94-98%.

Gas-Chromatography Mass Spectroscopy

Trimethylsilyl derivatives of sterols were prepared in glass tubes 75 mm x 2 mm and sealed prior to heating. The 7α-, 20- and 22-hydroxycholesterols as well as 7α-hydroxypregnenolone were converted to the trimethylsilyl ethers by dissolving in bis(trimethylsilyl)trifluoroacetamide and heating at 60°C for 60 mins. The 20,22-dihydroxycholesterol was dissolved in a mixture of pyridine, bis(trimethylsilyl)acetamide and trimethylchlorosilane (molar ratio 5:5:1) and heated at 120°C for 60 mins to obtain the tri(trimethylsilyl) ether. The trimethylsilyl derivatised sterols were chromatographed using a Hewlett Packard 402 gas chromatograph on a column 150 cms x 0.6 cms) containing 1% Desisil 300 on Chromasorb G with a column temperature of 300°C and a helium flow of 40 ml/min. The gas chromatograph was coupled through a Watson-Bieman separator, maintained at 300°C, to an A.E.I. MS12 mass spectrometer. Spectra were recorded at 20 eV with a block temperature of 280°C and scan speed of 16 seconds/decade. The data was acquired and processed by an A.E.I. DS30 data system.

Isoelectric Focusing

Analytical isoelectric focusing was performed on Ampholine PAG plates pH range 3.5-9.5, prepared thin layer polyacrylamide gels, in an LKB 2117 Multiphor electrophoresis unit using an LKB 2103 power supply. The pH gradient was established by a one hour pre-run at settings: 30W, 1500 V_{max} and 200 mA_{max} prior to the application of the

protein solutions to areas of gel corresponding to neutral pH. The protein samples were run for a further hour at the same settings. The pH gradient was determined by cutting gel pieces and homogenising in de-aerated distilled water. Gels were stained with Coomassie Brilliant Blue R-250 (Vesterberg (1972). Benzidine staining was by the method of Welton and Aust (1974).

Preparative flat bed electrofocusing in granulated Sephadex G75, was performed according to the method of Winters et al. (1975) using the LKB 2117-501 Ampholine Electrofocusing Kit. Typical instrument settings were 8W constant power, 1500 V_{max} with 20 mA_{max} and timed run 15 hr.

Optical Spectra

Absorption spectra were recorded on a Pye Unicam SP800 spectrophotometer fitted with a temperature regulated cell holder.

Anaerobic spectroscopy was performed in a 2.5 ml quartz cuvette with a tapered ground glass joint size C10. A jointed glass extension attachment, length 5 cm x 1 cm diameter was used above the cuvette and sealed with a size 17 Suba-seal. The joint between the cuvette and attachment was sealed at its widest diameter with a thin film of silicon grease. Anaerobiosis was obtained by equilibration of the cuvette contents with oxygen free nitrogen. Nitrogen of 'white spot' quality (British Oxygen Company) was passed through a pyrogallol solution and then through 1 M potassium phosphate buffer pH 7.4 to trap any alkali carried over. The pyrogallol solution was prepared by dissolving 15 gm of pyrogallol in 50 gm% w/v sodium hydroxide in distilled water pre-equilibrated with nitrogen. Nitrogen from these solutions was passed by means of thin teflon tubing and a long 18 gauge needle through the Suba-seal to the bottom of the cuvette. The cuvette contents were bubbled with nitrogen for 30 min and excessive frothing

was prevented by coating the tip of the needle with a thin film of Antifoam A Concentrate. Small vials with Suba-seal or rubber stoppers of a diaphragm type were used for stock solutions of NADPH or sodium dithionite and anaerobiosis obtained in the same manner as that for the cuvette.

The stock solutions of NADPH were 10 mM in 0.5% w/v sodium bicarbonate and that for sodium dithionite 10 mM in distilled water. Transfer of solutions from vials to cuvette was made with an air tight 10 µl syringe.

For experiments requiring carbon monoxide saturation of solutions, anaerobiosis of cuvettes and vials was attained as above and then bubbled with carbon monoxide for 15 min. The carbon monoxide was passed through the pyrogallol and phosphate buffer solutions prior to use.

Single and Multiple Turnover Cycles of Cytochrome $P450_{scc}$

($4\text{-}^{14}C$) cholesterol in acetone was evaporated onto the bottom of a vial with nitrogen gas. Cholesterol, stock solution 2 mM in propylene glycol, was added to the vial and stirred for 2 hr. The recovery of ($4\text{-}^{14}C$) cholesterol in the propylene glycol was 96-98% of initially added radioactivity. The ($4\text{-}^{14}C$) cholesterol was purified immediately before use by thin layer chromatography on silica gel H (developing solvent; di-isopropyl ether - petroleum spirit - acetic acid, 70:30:2, by volume). The region corresponding to a standard cholesterol sample was eluted from the thin layer chromatography plate using re-distilled acetone.

Deoxy $1\alpha 2\alpha(n)\text{-}^{3}H$ corticosterone was pre-equilibrated with deoxycorticosterone, 2 mM in propylene glycol, in the same manner. Deoxy $1\alpha 2\alpha(n)\text{-}^{3}H$ corticosterone was purified immediately before use by thin layer chromatography on silica gel H (developing solvent

chloroform:ethyl acetate, 8:2 by volume). The region corresponding to a standard deoxycorticosterone sample was eluted from the thin layer chromatography plate using redistilled acetone.

(4-^{14}C)7α-hydroxycholesterol was pre-equilibrated with 7α-hydroxycholesterol, final concentration 2 mM in propylene glycol. (4-^{14}C) 7α-hydroxycholesterol was purified prior to use by thin layer chromatography on silica gel H (developing solvents; benzene: ethyl acetate::7:13 by volume).

Cholesterol or deoxycorticosterone, prepared as above and of known specific radioactivity, was added to low spin substrate depleted cytochrome $P450_{scc}$ in 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA and allowed to incubate 15 hr at 20°C. The substrate was added to a two fold molar excess to cytochrome P450 and the radioactivity prior adjusted such that 0.1 μCi was added to 2 ml cytochrome $P450_{scc}$ solution.

Adrenodoxin was added to a 1:1 molar ratio with cytochrome $P450_{scc}$ substrate complex. A typical reaction solution was 2.2 ml cytochrome $P450_{scc}$ substrate complex (stock solution 17 nmoles cytochrome P450/ml) and 0.23 ml adrenodoxin (153 nmoles adrenodoxin/ml). The reaction temperature was 37°C and incubations performed in a 2.5 ml spectrophotometer cuvette.

Solvent Extraction of Incubations

The contents of a cuvette were poured into a 'Quickfit' joined test tube and the cuvette rinsed with a total volume of 10 ml methanol; the methanol rinses were added to the contents of the tube. Chloroform 20 ml was added to the tube followed by 30 ml water and the organic and aqueous phases partitioned by centrifugation. The aqueous phase was discarded and the organic phase carefully decanted into a boiling tube leaving the protein, which had formed an interface with organic and aqueous phases.

The protein was re-extracted in a stoppered tube with 20 ml hot ethyl acetate and the protein removed by centrifugation. The ethyl acetate was decanted and the protein re-extracted with the same solvent. The combined organic solvent extracts were taken to dryness in a warm water bath and evaporated by a stream of air. Each residue was dissolved in 0.2 ml of chloroform.

Thin Layer Chromatography

The residue dissolved in chloroform was transferred to a thin layer plate (28 cm x 6.5 cm) coated with silica gel H. This process was repeated three times for each residue resulting in 95-98% of the extracted radioactivity being transferred to the plate. Standard non-radioactive steroids were also applied at the origin. The thin layer plates were developed to the full length of the plate in the solvent system; diisopropyl ether:petroleum spirit:acetic acid, 70:30:2 by volume. This solvent system (Simpson and Boyd (1966) has the property of separating the postulated mono- and dihydroxylated intermediates of side chain cleavage of cholesterol from cholesterol and from the C21 steroids.

Deoxycorticosterone and corticosterone were separated by thin layer chromatography on silica gel H (developing solvents; chloroform: ethyl acetate::8:2 by volume).

7α-hydroxycholesterol and 7α-hydroxypregnenolone were separated by thin layer chromatography on silica gel H (developing solvents; benzene:ethyl acetate::7:13 by volume).

Determination of Radioactivity

Thin layer plates were scanned in a Panax RTLS 1A thin layer radioactive scanner. Silica gel was scraped from the plates into vials and 5 ml of scintillant added. The scintillation fluid was prepared by adding 20 gm 2,5 diphenyloxazole and 150 gm 1,4-Di 2-(5-phenyloxazolyl)

benzene dissolved in 250 ml methanol to 4750 ml of toluene. Radioactivity was then quantitatively determined in a Packard Tri-Carb liquid scintillation spectrometer. The efficiency of counting ^{14}C disintegrations was 81% and ^{3}H 30%.

CHAPTER 3

PARTIAL PURIFICATION AND PROPERTIES OF CYTOCHROME $P450_{scc}$

INTRODUCTION

Cytochrome P450 behaves as an integral protein of adrenal cortex mitochondria according to the classification of Singer and Nicolson (1972). Osmotic disruption and sub-mitochondrial fractionation by Satre et al. (1969) and Sottocasa and Sandri (1970) suggested that cytochrome P450 and 11β-hydroxylase activity of bovine adrenal mitochondria occurred in the inner membrane fraction. Similar conclusions were reached by Yago and Ichii (1969) for the cholesterol side chain cleavage system. The ready release of other components of the mitochondrial hydroxylase system in soluble form, namely adrenodoxin and adrenodoxin reductase, by ultrasonic disruption of mitochondria suggests that these proteins are present in the soluble matrix or loosely associated with the mitochondrial membrane (Kimura et al.(1976).

The use of optical difference spectroscopy to study ligand interactions with cytochrome P450 developed in the main from investigations into the nature of this haemoprotein in liver microsomes. Liver microsomes contain a variety of distinct cytochrome P450s with individual catalytic specificities (van der Hoeven et al. (1974)), cytochrome b_5 and are invariably contaminated by haemoglobin; absolute spectral changes may be difficult to appreciate in this mixture of haemoproteins. Optical difference spectra of cytochrome P450 can be classified into three groups:

a) Type I spectral change characterised by a peak of absorbance at around 385 nm and an absorbance trough at 420 nm.

b) An inverse Type I spectral change which is the mirror of the Type I spectral change (λ max 420 nm, λmin 385 nm).

c) Type II spectral change characterised by a broad trough between 390 and 410 nm and an absorbance peak varying between 425 nm and 435 nm. Amines interact with cytochrome P450 and optical

difference spectra vary between two extremes termed Type IIa (λ max 425 nm, λ min 390 nm) and Type IIb (λ max 432 nm, λ min 410 nm) (Schenkman et al. (1967); Calabrese et al. (1968)).

Electron paramagnetic and magnetic susceptibility studies of cytochrome $P450_{cam}$ (Tsai et al. (1970)) have established that cytochrome P450 can exist as a high spin cytochrome (Fe^{+++} haem, S = 5/2), as a low spin cytochrome (Fe^{+++} haem, S = 1/2) or more frequently in a mixed spin state where the electron paramagnetic spectra show distinct high and low spin components. Absolute optical spectra of purified cytochrome $P450_{cam}$ have been similarly resolved into spectra of a high spin cytochrome (λ max 392, 540 and 645 nm) and a low spin cytochrome (λ max 416, 535 and 565 nm). Mixed spin states are more commonly observed in which the spectra are derived additively from the individual spectral components, Peterson (1971). The basis of the four categories of optical difference spectra has become evident from electron paramagnetic studies of ligand interactions with cytochrome P450 (Stern et al. (1973); Grasdalen et al. (1975)). In summary, a Type I spectral change reflects an increase in the high spin character of the haemoprotein upon binding of the ligand whereas an inverse Type I change reflects the opposite. A Type IIa difference spectrum reflects a change from a high spin complex to that of a low spin complex of the cytochrome in which the haem bonding is perturbed. The Type IIb difference spectrum reflects a change from the spectrum of a low spin cytochrome P450 to the spectrum of a perturbed low spin state of the cytochrome (Jefcoate (1978)).

The concept of separate forms of cytochrome P450 in adrenal cortex mitochondria has developed, as a consequence of various observations. Cytochrome P450 has the property of binding steroids to give characteristic optical and EPR spectra. For example a Type I optical difference

(max 385 nm, min 420 and 570 nm) characterises the change from low spin substrate depleted enzyme to the high spin enzyme-substrate complex (Schenkman et al. (1967)), and can be obtained by addition of 11-deoxycorticosterone, a substrate for the 11β-hydroxylase enzyme,to adrenal cortex mitochondria. A spectral change, the mirror image of the above, is produced by addition of pregnenolone or (20S)-20-hydroxycholesterol. These spectral changes are non-additive and Whysner et al. (1969) suggested that they can be interpreted as involving distinct cytochrome P450 binding sites. Thus it appears that the Type I optical change produced by 11-deoxy-corticosterone is due to the formation of a haemoprotein-substrate complex with a form of cytochrome P450 involved in 11β-hydroxylation, which in the absence of added substrate was largely in the free form. However in the case ofthe haemoprotein concerned with cholesterol side chain cleavage the inverted Type I spectrum produced by pregnenolone is due to the displacement of endogenous substrate, cholesterol, from this cytochrome P450. The cholesterol side chain cleavage cytochrome P450 occurs at least in bovine adrenal cortex mitochondria largely in a substrate bound form. Cheng and Harding (1973) showed that when cholesterol was removed from their preparation, (20S)-20-hydroxycholesterol no longer produced an inverted Type I spectrum and only did so when cholesterol was added to the preparation. The substrate cholesterol produced a characteristic Type I spectrum when added to the preparation. The EPR spectrum of bovine adrenal mitochondria shows both low spin ferric and high spin ferric forms to be present. Addition of (20S)-20-hydroxycholesterol causes a decrease in the g = 8.05 signal due to a decrease in the high spin cytochrome P450 and an increase in the low spin form. When deoxycorticosterone is added in the presence of (20S)-20-hydroxycholesterol, another high spin species is formed with a

g value of 7.9 (Jefcoate et al. (1973)). The conclusion that the two species of cytochrome P450 which catalyse cholesterol side chain cleavage and 11β-hydroxylation are distinct entities was proved by the demonstration of their physical separation (Jefcoate et al. (1970)). The separation of the specific cytochrome P450 (cytochrome $P450_{scc}$) associated with the side chain cleavage of cholesterol from that associated with the 11β-hydroxylation of deoxycorticosterone (cytochrome P450 11β) has been confirmed by Ramseyer and Harding (1973) and Shikita and Hall (1973).

The solubilisation and purification of cytochrome $P450_{scc}$ from bovine adrenal mitochondria has been attempted by a number of investigators (Ramseyer and Harding (1973); Shikita and Hall (1973); Horie and Watanabe (1975); Takemori et al. (1975) and Wang and Kimura (1976)). These preparations are of low specific content in terms of nmoles cytochrome P450/mg protein and have significant contamination by both cholesterol and detergent. The resolution of cytochrome $P450_{scc}$ from other membrane components particularly the substrate cholesterol is fundamental to the further understanding of the enzyme. The primary objectives for purification of cytochrome $P450_{scc}$ may be summarised as follows:

a) separation of cytochrome $P450_{scc}$ from cytochrome P450 11β;

b) the development of techniques for removal of lipid and in particular substrate cholesterol from cytochrome $P450_{scc}$;

c) to obtain cytochrome $P450_{scc}$ free from adrenodoxin and adrenodoxin reductase;

d) to render cytochrome $P450_{scc}$ soluble in an aqueous media free from detergents;

e) to stabilise cytochrome $P450_{scc}$ during purification by the use of ligands, and

f) the development of chromatographic procedures for purification of cytochrome $P450_{scc}$ to a high specific content.

RESULTS AND DISCUSSION

Isooctane Extraction of Lyophilised Adrenal Cortex Mitochondria

Lyophilised adrenal cortex mitochondria were suspended by homogenisation, 20 mg/ml protein, in 100 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA and 1 mM 4-phenylimadazole (Fig. 3.1). The homogenate was centrifuged at 105,000 g for 60 min and the supernatant discarded; the pellet was resuspended by homogenisation in the same volume of buffer and centrifugation repeated, again discarding the supernatant. The use of 100 mM potassium phosphate buffer pH 7.4 was found to give optimal extraction of soluble proteins with minimal losses of cytochrome P450. The specific content of cytochrome P450 increased to 1.75 nmoles/mg protein (Table 3.1).

Many methods are available to dissociate integral proteins from membranes and include the use of detergents, proteases, lipases, organic solvents and protein denaturants. The use of iso-octane extraction to partially remove lipid from mitochondrial membrane containing cytochrome P450 was developed for use with this haemoprotein. The method allows removal of the organic solvent by phase partition and recoveries of cytochrome P450 are acceptable. Mild sonication was included to facilitate the development of an emulsion and increase the effectiveness of extraction. This method allows removal of ubiquinones, carotenoids, approximately 30% of the phospholipid and about 45% of the cholesterol. Although the specific content of cytochrome P450 is not increased by iso-octane extraction this step is required to allow separation of cytochrome P450 with cholesterol side chain cleavage activity from cytochrome P450 with 11β-hydroxylase activity during the subsequent ammonium sulphate fractionation in the

TABLE 3.1. Recoveries of protein and cytochrome P450 during partial purification of cytochrome $P450_{scc}$

The specific content of cytochrome $P450_{scc}$ is expressed in nmoles cytochrome P450/mg protein. Protein and cytochrome P450 concentrations were determined as in Methods.

	Protein mgs	Cytochrome P450 nmoles	nmoles cytochrome P450/mg protein	Percentage recovery cytochrome P450
Lyophilised mitochondria	2122	2122	1.0	100
105,00 g mitochondrial pellet	1139	1994	1.75	94
Lyophilised isooctane interlayer	942	1697	1.8	80
35-50% $(NH_4)_2SO_4$ fraction	518	933	1.8	44
Butanol-acetone powder	339	746	2.2	33
105,000 g supernatant of butanol-acetone powder	122	537	4.4	24
35-50% $(NH_4)_2SO_4$ fraction	74	403	5.5	19
Calcium phosphate gel eluate	29	297	10.2	14

presence of sodium cholate (Jefcoate et al. (1970)). The 105,000 g pellet was resuspended by homogenisation in the above buffer at 20 mg protein/ml and stirred vigorously with a magnetic stirrer. An equal volume of iso-octane was added dropwise over 10 min into the vortex of the suspension. This emulsion was sonicated using an Ultrasonic Rapidis 150 sonicator with the specifications: frequency 20kc, amperage 3, using a 9 millimetre probe at setting 9 for a total of 2.5 min. Sonication was performed in an ice cooled 250 ml beaker for 30 sec periods with intervals of 30 sec. The sonicated emulsion was gently stirred for 30 min then centrifuged at 30,000 g for 20 min. The centrifuged emulsion separated into three phases; a yellow upper phase of iso-octane; a lower aqueous phase and a middle dark brown band between the upper and lower phases and containing the bulk of the protein. The iso-octane phase was discarded and the middle and lower phases re-homogenised together. This extraction process was repeated and after centrifugation the upper and lower phases were discarded. The middle phase, which will be referred to as the iso-octane interlayer, containing the bulk of cytochrome P450 and other proteins was rehomogenised in a minimum volume of distilled water and lyophilised (Fig. 3.1). The lyophilised powder was stored at $-20^{\circ}C$ in the flask used for lyophilisation.

Ammonium Sulphate Fractionation of Lyophilised Iso-Octane Interlayer

The lyophilised iso-octane interlayer was suspended by homogenisation, 20 mg protein/ml, in 100 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA and 1 mM 4-phenylimidazole. Sodium cholate, stock solution 100 mg/ml in the above buffer, was added dropwise over 10 min to the stirred iso-octane interlayer suspension (Fig. 3.1). The final concentration of sodium cholate was 0.5 mg/mg protein and after addition of this detergent the suspension was gently stirred for 30 min. The

concentration of sodium cholate used was optimal for the solubilisation of cytochrome P450 from mitochondrial membrane and similar to the values used by Mitani and Horie (1969). Ammonium sulphate, stock solution 80% saturated in 100 mM potassium phosphate buffer pH 8.0 containing 1 mM EDTA, was then added over 30 min by means of a peristaltic pump to produce a solution which was 35% saturated with respect to ammonium sulphate. This solution was centrifuged at 30,000 g for 30 min. The pellet was discarded and to the supernatant, ammonium sulphate solution was added over 30 min to produce a solution which was 50% saturated with respect to ammonium sulphate. This solution was centrifuged at 30,000 g for 20 min. The pellet from this centrifugation was resuspended in a minimum volume of 10 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA and 1 mM 4-phenylimadazole and dialysed against 3 litres of 10 mM potassium phosphate buffer pH 7.4 for 15 hr, followed by dialysis against 3 litres of distilled water for 3 hr (Fig. 3.1). The dialysate was lyophilised and will be referred to as the 35-50% ammonium sulphate fraction. This fraction was stored at -20^{o}C in the flask used for lyophilisation. The ammonium sulphate fraction which precipitated between 25-35% saturation showed high 11β-hydroxylase and low cholesterol side chain cleavage activity. Conversely the fraction which precipitated between 35-50% ammonium sulphate saturation showed the reverse pattern. The 25-35% ammonium sulphate fraction was predominantly a low spin haemoprotein showing maximal binding with 11-deoxycorticosterone whereas the 35-50% fraction was predominantly a high spin haemoprotein and bound pregnenolone in an inverse Type I manner (Jefcoate et al. (1970)). This preparation of cytochrome $P450_{scc}$ gave useful information relating to spectral and EPR changes (Simpson et al. (1971); Jefcoate et al. (1973)). However the preparation was of low specific content, 1.8 nmoles/mg protein (Table 3.1) and on

dialysis to remove ammonium sulphate the preparation became turbid and approximately 50% of the cytochrome precipitated on centrifugation at 105,000 g for 60 min. The turbidity could be reversed by readdition of sodium cholate suggesting that solubility of the preparation was dependent on the detergent present. Similarly on titration of the preparation with pregnenolone, biphasic, inverse Type I binding plots were obtained, whereas uniphasic binding was observed in intact mitochondria. On centrifugation of this solution at 105,000 g x 60 min, the pellet and supernatant gave uniphasic spectral binding plots with pregnenolone but exhibited different spectral dissociation constants. The cytochrome $P450_{scc}$ isolated was predominantly a high spin substrate bound form with a cholesterol content of 8-10 fold molar excess (Table 3.2) and was unsuitable for kinetic studies of substrate binding.

Butanol-Acetone Extraction of the Lyophilised 35-50% Ammonium Sulphate Fraction

The lyophilised 35-50% ammonium sulphate fraction was stored at $-20^{o}C$ and without warming, 1 ml of butanol at $-20^{o}C$ was added for each 4 mg protein. The butanol-protein suspension was homogenised with an all glass homogeniser and centrifuged at 20,000 g for 5 min. in a rotor and centrifuge pre-cooled to $-20^{o}C$. The supernatant was discarded and the pellet resuspended by homogenisation in acetone at $-20^{o}C$. The acetone-protein suspension was rapidly filtered on a Buchner funnel and washed with three volumes of acetone. The butanol-acetone extracted powder retained on filtration was immediately dried for 1 hr in a vacuum desiccator under continuous suction. The butanol-acetone extracted powder was used immediately after drying (Fig. 3.1). The solvent n-butanol has been used extensively for removal of lipid particularly phospholipid from protein (Morton (1955)) and combined with acetone extraction at sub-zero temperature has proved particularly useful for the further resolution of cytochrome $P450_{scc}$. The

recovery of cytochrome $P450_{scc}$ after this procedure is approximately 80% and the specific content increases (Table 3.1). The phospholipid, cholesterol and cholate contents are all reduced. Associated with the reduction of the cholesterol content the cytochrome is now in a predominantly low spin state (Fig. 3.2).

Potassium Phosphate Buffer Extraction of a Butanol-Acetone Extracted Powder

The butanol-acetone extracted powder was suspended by homogenisation in 100 mM potassium phosphate buffer pH 7.4 containing 5 mM D,L-dithiothreitol. The homogenate was centrifuged at 105,000 g for 60 min; the supernatant carefully collected and the pellet discarded (Fig. 3.1). The protein concentration of the homogenate was varied from 4 to 12 mg/ml depending on the concentration of cytochrome P450 required in the 105,000 g supernatant.

The 105,000 g x 60 min supernatant of a butanol-acetone powder shows an increase in specific content of cytochrome compared to the pellet and the original powder (Table 3.1). No deoxycorticosterone binding can be detected in this supernatant and no 11β-hydroxylase activity demonstrated.

Cytochrome $P450_{scc}$ is now substrate depleted with a cholesterol:cytochrome $P450_{scc}$ molar ratio 0.01:1 (Table 3.2) and further purification of this cholesterol depleted cytochrome $P450_{scc}$ was associated with marked loss of this haemoprotein. Addition of cholesterol or pregnenolone, the product of the cholesterol side chain cleavage reaction, stabilised the cytochrome $P450_{scc}$ to further purification (Table 3.5) and this protective effect was observed with ligands to the haemoprotein other than substrate or product (Table 3.5).

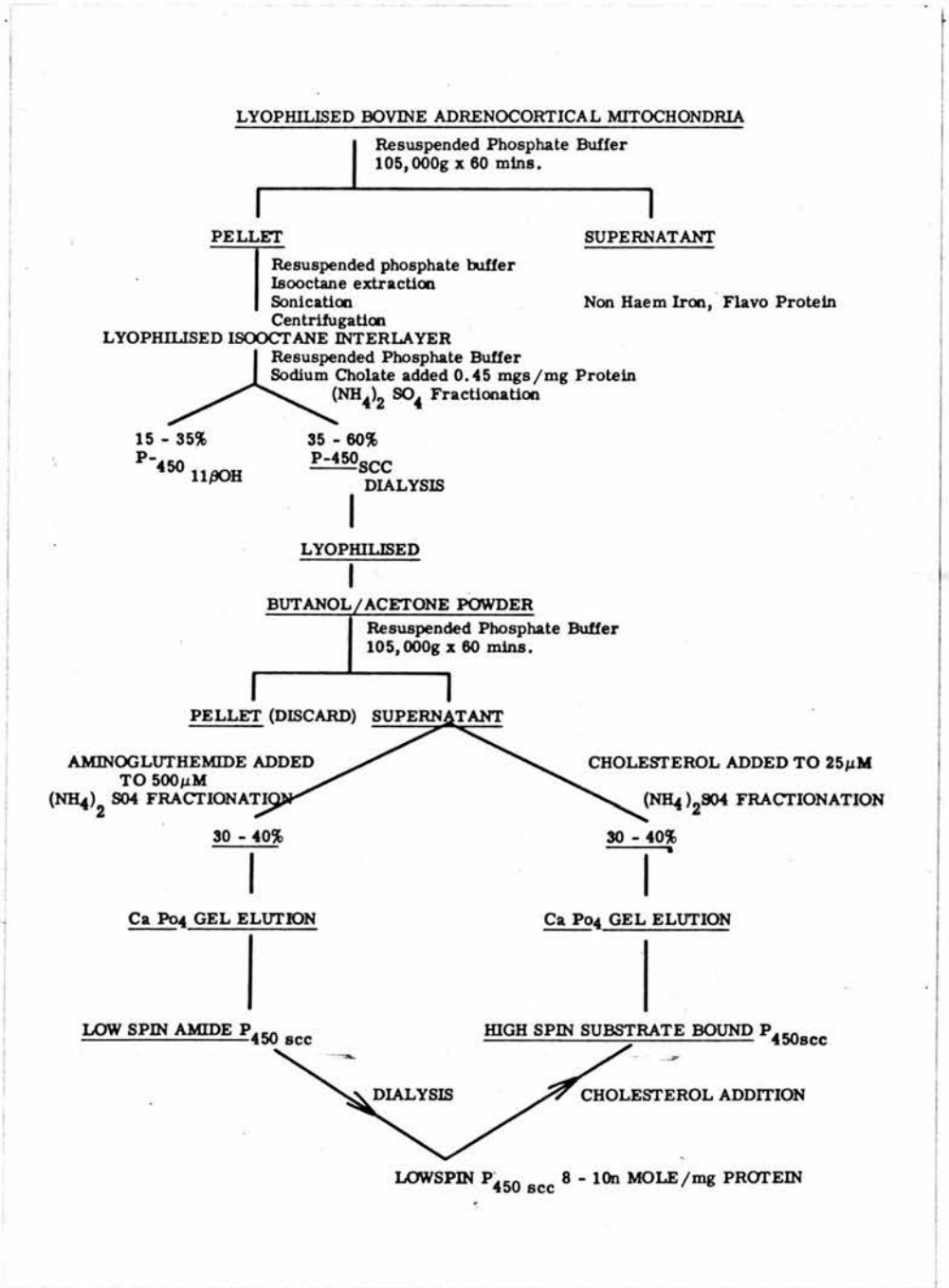


Fig. 3.1. SCHEME FOR THE PARTIAL PURIFICATION OF CYTOCHROME P450$_{scc}$ FROM BOVINE ADRENAL CORTEX MITOCHONDRIA

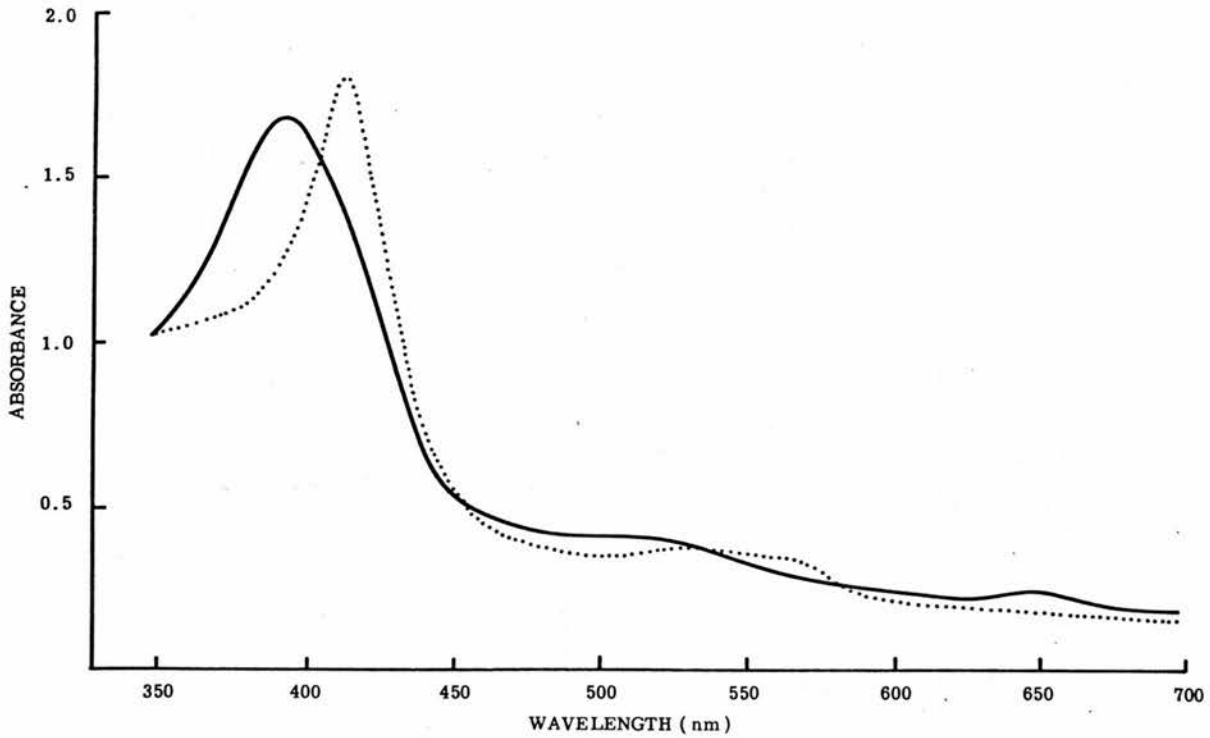


Fig. 3.2. ABSOLUTE SPECTRA OF CYTOCHROME $P450_{scc}$ BEFORE AND AFTER BUTANOL-ACETONE EXTRACTION

Cytochrome $P450_{scc}$ is predominantly in a high spin state prior to butanol-acetone extraction with absorption maxima at 392 and 648 nm. The cholesterol to cytochrome $P450_{scc}$ molar ratio is 8:1. After extraction the absorption maxima are 416, 535 and 568 nm with a cholesterol to cytochrome $P450_{scc}$ molar ratio of 0.01:1. Cytochrome $P450_{scc}$ concentration was adjusted, in both cases, to 17.2 nmoles/ml on the basis of the carbon monoxide difference spectrum. The spectra were recorded at 20°C and the cytochrome was suspended in 10 mM potassium phosphate buffer pH 7.4.

_____ cytochrome P450 spectrum prior to butanol-acetone extraction.

------- cytochrome P450 spectrum after butanol-acetone extraction.

Ammonium Sulphate Fractionation of the 105,000 g Supernatant of a Butanol-Acetone Extracted Powder

The two ligands most commonly employed were aminoglutethimide and 4-phenylimidazole at 0.5 mM and 1 mM concentrations respectively. The stock solution of aminoglutethimide was 10 mM in propylene glycol and that of 4-phenylimidazole was 45 mM in propylene glycol. To the stirred 105,000 g supernatant extract, ammonium sulphate was added dropwise over 45 min to a saturation of 35% and centrifuged at 30,000 g for 20 min. The stock solution of ammonium sulphate was 80% saturated in 100 mM potassium phosphate buffer pH 8.0, 1 mM EDTA. The small pellet formed on centrifugation was discarded. Ammonium sulphate solution was added to the supernatant over 45 min to a saturation of 50% and centrifuged at 30,000 g for 20 min. The pellet formed was resuspended in a minimum volume of 10 mM potassium phosphate buffer pH 7.4 with 1 mM EDTA and containing either 0.5 mM aminoglutethimide or 1 mM 4-phenylimidazole (Fig. 3.1). The resuspended pellet was dialysed for 3 hr against 3 litres of 10 mM potassium phosphate buffer pH 7.4. The specific content of cytochrome $P450_{scc}$ increased with minimal loss of cytochrome (Table 3.1).

Calcium Phosphate Gel Fractionation

Calcium phosphate gel was washed initially with distilled water and then resuspended in a thin slurry with 10 mM potassium phosphate buffer pH 7.4. The 35-50% ammonium sulphate fraction of the 105,000 g supernatant of a butanol-acetone powder was resuspended in 10 mM phosphate buffer pH 7.4, stirred and a minimal volume of calcium phosphate gel added (Fig. 3.1). When concentrated, cytochrome P450 is dark brown in colour and thus the calcium phosphate gel was titrated into the cytochrome solution until all the coloured protein was adsorbed on a minimal amount of gel. The gel was then centrifuged at 3,000 g for 5 min and the

supernatant decanted. The pellet was gently stirred with a higher molarity phosphate buffer for 20 min and the centrifugation repeated. During the initial experiments with this technique, increments were of 20 mM potassium phosphate buffer. However when the elution pattern of protein and cytochrome P450 was realised the gel was washed with 100 mM potassium phosphate buffer after the initial wash with 10 mM potassium phosphate buffer and cytochrome P450 subsequently eluted with 200 mM buffer. The cytochrome P450 eluted was dialysed against 3 litres of 10 mM potassium phosphate buffer pH 7.4 to give a preparation of cytochrome $P450_{scc}$ with a specific content of 8-10 nmoles/mg protein (Table 3.1).

The elution pattern changes with the ligand employed to stabilise the cytochrome P450. In the example shown (Fig. 3.3), cytochrome $P450_{scc}$ - aminoglutethimide elutes maximally within the range 100-200 mM potassium phosphate buffer whereas cytochrome P450-cholesterol complex elutes maximally in the range 150-250 mM potassium phosphate. Overall yields and specific contents of cytochrome $P450_{scc}$ for the last two stages of purification are marginally greater for the cytochrome stabilised with aminoglutethimide than stabilised with cholesterol.

Cholesterol

The cholesterol content of the preparation is reduced as the purification of cytochrome $P450_{scc}$ proceeds (Table 3.2). The stages associated with the most marked cholesterol reductions are the iso-octane extraction of lyophilised mitochondria, the ammonium sulphate fractionation of iso-octane interlayer in the presence of sodium cholate and the butanol-acetone extraction of 35-50% ammonium sulphate fraction. Associated with the changes in cholesterol content cytochrome $P450_{scc}$ is predominantly high spin until the butanol-acetone extraction when the cytochrome becomes predominantly low spin (Fig. 3.2). However

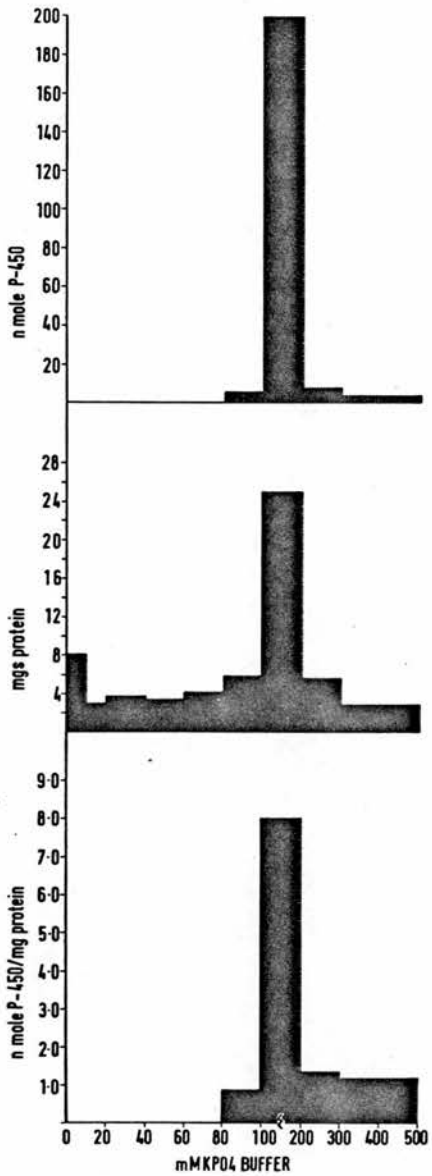


Fig. 3.3. ELUTION PATTERN OF PROTEIN AND CYTOCHROME $P450_{scc}$-AMINOGLUTETHIMIDE COMPLEX FROM CALCIUM PHOSPHATE GEL WITH INCREASING POTASSIUM PHOSPHATE BUFFER pH 7.4 CONCENTRATION

The results are also expressed in terms of specific content, nmoles cytochrome $P450_{scc}$/mg protein.

addition of cholesterol to this low spin haemoprotein restores the haemoprotein to the high spin states.

Sodium Cholate

The concentration of sodium cholate, added to solubilise cytochrome P450 from the lyophilised iso-octane interlayer, was reduced as the preparation proceeded (Table 3.3). The purification steps associated with the most marked reduction in sodium cholate were the dialysis of the 35-50% ammonium sulphate fraction and the butanol-acetone extraction. The concentration of sodium cholate in the 105,000 g supernatant fraction of a butanol-acetone powder extract was in three fold molar excess to cytochrome $P450_{scc}$. Similar reductions in cholate concentration have been reported by Autor et al. (1973) from cholate solubilised liver microsomal cytochrome P450.

Cytochrome $P450_{scc}$ behaves as an integral protein of mitochondrial membrane (Singer and Nicolson (1972)) and as a rule such proteins are not very soluble in aqueous solutions. Cytochrome $P450_{scc}$, after butanol-acetone treatment, is phospholipid and detergent depleted but is retained in the 105,000 g x 60 min supernatant of a phosphate buffer extract. The cytochrome $P450_{scc}$ from this supernatant behaves as an aggregate of apparent molecular weight 200,000 and one possible explanation of this solubility could be that by mutually covering hydrophobic domains, individual proteins form a complex with sufficient polar surface to remain soluble in water (Helenius and Simons (1975)).

Detergents both ionic and non-ionic can act as denaturants to cytochrome P450 (Mason et al. (1965)). Cytochrome $P450_{scc}$ is exposed to a concentration of sodium cholate well above the critical micellar concentration (Carey and Small (1970)) during the solubilisation of the iso-octane interlayer. However when cytochrome $P450_{scc}$ is

TABLE 3.2 NADPH adrenodoxin reductase activities and cholesterol: cytochrome P450 molar ratios during purification of cytochrome $P450_{scc}$

Determination of cytochrome P450 and cholesterol concentrations were as described in Methods. NADPH adrenodoxin reductase activity was determined by the method of Omura et al. (1966) and is expressed as nmoles 2,6-dichlorophenol-indophenol reduced/min/nmole cytochrome P450.

	nmoles 2,6-dichlorophenol reduced/min/nmole cytochrome P450	nmole cholesterol/nmole cytochrome P450
Lyophilised mitochondria	200	45
Iso-octane interlayer	19	25
35-50% $(NH_4)_2SO_4$ fraction	10	8
105,000 g supernatant of butanol-acetone powder	8	0.01

TABLE 3.3 Sodium cholate:cytochrome P450 molar ratios during purification of cytochrome $P450_{scc}$. Cholate and cytochrome P450 concentrations were determined as in Methods

	Molar ratio sodium cholate: cytochrome $P450_{scc}$
Iso-octane interlayer - (0.5 mg cholate/mg protein)	655:1
Dialysed 35-50% $(NH_4)_2SO_4$ fraction	24:1
105,000 g supernatant of butanol-acetone powder	3:1

depleted of this added cholate it retains enzymatic and spectral properties similar to those of cytochrome P450 in intact mitochondria.

NADPH Adrenodoxin Reductase

The most substantial decrease in NADPH-adrenodoxin reductase activity was noted on washing lyophilised mitochondria. Residual activity in the 105,000 g supernatant of a butanol-acetone powder represents a twenty-five fold decrease in activity compared to lyophilised mitochondria (Table 3.2).

Adrenodoxin

Adrenodoxin is usually determined by examination of the characteristic electron paramagnetic resonance (EPR) spectrum of the reduced protein at liquid nitrogen temperature. Under these conditions adrenodoxin has signals in the first derivative spectrum with g values at 1.94 and 2.03 (Fig. 3.4). The adrenodoxin in adrenocortical mitochondria can be quantitated from the height of the signal of the EPR spectrum at g 1.94 by comparison with a highly purified adrenodoxin preparation (Estabrook et al. (1973)). The EPR spectra of the 'adrenodoxin like' iron sulphur proteins are perturbed, however, if the preparations contain large amounts of the respiratory chain iron sulphur proteins, since these proteins also exhibit EPR signal in the same region of the spectrum. Signals in the 1.86-1.92 region are indicative of such respiratory chain iron sulphur proteins (Orme-Johnson (1973)). This overlap in signal position makes quantitative determination of adrenodoxin difficult when levels are low and the predominant signal appears to be that of the iron sulphur protein of respiratory chain origin. Similar problems of iron sulphur protein quantitation have been observed in the determination of testodoxin levels in testis mitochondria (Mason et al. (1973)).

TABLE 3.4 The molar ratios of iron sulphur protein:cytochrome $P450_{scc}$ at later stages in the purification of cytochrome $P450_{scc}$

The contamination of cytochrome $P450_{scc}$ by iron sulphur protein was reduced to minimal amounts by adequate washing of the lyophilised mitochondria with 100 mM potassium phosphate buffer pH 7.4 and by the inclusion of a 50% ammonium sulphate precipitation on a sodium cholate solubilised iso-octane interlayer. The individual experiments show variation in (1) concentration, protein/ml, of resuspended lyophilised mitochondria; (2) number of times a resuspension was washed and, (3) whether the 50% ammonium sulphate fractionation was included.

Experiment	A	B	C
Concentration mitochondria mg/ml	40	20	20
Number mitochondrial washes	1	2	2
Inclusion of 50% $(NH_4)_2SO_4$ fractionation	-	-	+
	Molar ratio iron sulphur protein: cytochrome $P450_{scc}$		
Butanol-acetone powder	1.1	1:12.5	1:100
105,000 g supernatant of butanol-acetone powder	1:0.58	1:20	<1:100
105,000 g pellet of butanol-acetone powder	1:22	1:9	<1:100

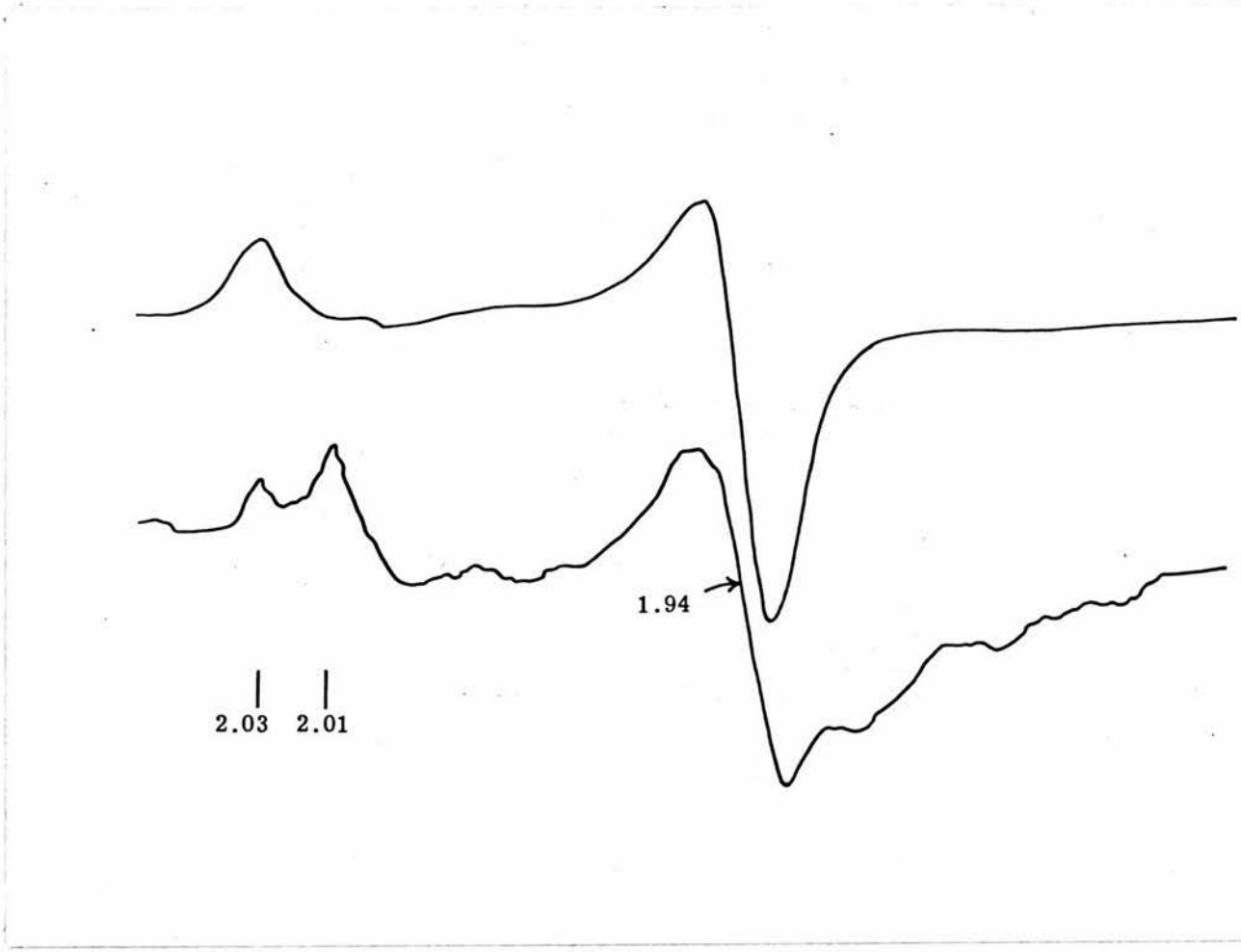


Fig. 3.4. THE ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF IRON SULPHUR PROTEINS DURING PURIFICATION OF CYTOCHROME $P450_{scc}$

Samples were suspended in 200 mM Tris-chloride buffer pH 7.4 and reduced with sodium dithionite. Sample A upper trace, a 105,000 g supernatant fraction extracted from butanol-acetone powder (expt. A, Table 3.4) containing 6.3 nmoles cytochrome P450/ml. Sample B lower trace, a butanol-acetone powder (expt. B, Table 3.4) containing 34.2 nmoles cytochrome P450/ml cytochrome $P450_{scc}$. The instrument settings were temperature $-172^{o}C$, modulation amplitude 12.59, microwave power 50 mW, modulation frequency 100 kHz and microwave frequency 9.14 GhZ. Sample A represents the EPR signal where the iron sulphur protein is in the main adrenodoxin and sample B represents significant contamination of the adrenodoxin with iron sulphur protein of respiratory chain origin.

Adrenodoxin is relatively loosely bound in adrenocortical mitochondria and can be released into a supernatant fraction on centrifugation by prior sonication or lyophilisation leaving most of the cytochrome P450 bound to membrane in the pellet. The data presented in Table 3.4 emphasises the importance of homogenisation and centrifugation of lyophilised mitochondria in excess phosphate buffer and the effect of the 50% saturated ammonium sulphate fractionation step, as necessary to further reduce adrenodoxin levels at a later stage in the preparation. These procedures reduce contamination of cytochrome $P450_{scc}$ by iron sulphur proteins to less than 1% on a molar basis. Adrenodoxin is relatively more soluble than the respiratory chain iron sulphur proteins as can be observed in expt. A, Table 3.4, when adrenodoxin is the main contaminant and the molar ratio of iron sulphur proteins cytochrome $P450_{scc}$ changes from 1:1 in a butanol-acetone powder to 1:0.58 in the 105,000 g supernatant fraction of a butanol-acetone powder extract. In expt. B, Table 3.4, by contrast when the respiratory chain iron sulphur proteins are a predominant contaminant the molar ratio of iron sulphur protein:cytochrome $P450_{scc}$ changes from 1:12.5 in a butanol-acetone powder to 1:20 in the 105,000 g supernatant fraction of a butanol-acetone powder extract.

Protective Ligands

Cytochrome P450 in the ferric, substrate depleted form has a Soret absorption band at 416 nm. Upon reduction the Soret band of the ferrous form moves to 411 nm. Upon addition of carbon monoxide to this reduced cytochrome P450 preparation the Soret band shifts to 446 nm. An enzymatically inactive derivative of the cytochrome forms on treatment of cytochrome P450 with mercurial compounds, acetone, sodium deoxycholate or urea (Mason et al. (1965)). This derived

cytochrome has a ferrous carbon monoxide Soret absorption at 420 nm. In general, all these biologically inactive forms are termed cytochrome P420. However such cytochrome P420s were not observed as a major degradation product during the purification of cytochrome $P450_{scc}$.

Another cytochrome P450 derivative has been observed with the appearance of a Soret absorption band at 412 nm and ferrous absorption band at 422 nm but failing to bind carbon monoxide (Fig. 3.5) when cytochrome P450 is treated by a wide variety of agents, high salt concentration, potassium superoxide, organic solvent, prolonged exposure to gel filtration or ionic exchange chromatography. This product cannot be re-converted to cytochrome P450 or cytochrome P420 by sulphydryl groups such as 10 mM dithiothreitol or cysteine or using 20% glycerol or a combination of thiols and glycerol.

This degraded form of cytochrome P450 was observed initially as a result of ammonium sulphate fractionation of the 105,000 g supernatant fraction of a butanol-acetone powder extract. Addition of cholesterol to such a preparation prior to ammonium sulphate fractionation prevents the production of such a haemoprotein and this is interpreted as a protective effect.

Aminoglutathimide is an inhibitor of the cholesterol side chain cleavage reaction (Kahnt and Neher (1966)) and forms a distinct spectral complex with cytochrome $P450_{scc}$ (Jefcoate et al. (1973). Mahaffee et al. (1974)) have shown the inhibitory effect of aminoglutethimide on the cholesterol side chain cleavage reaction can be reversed by washing mitochondria prepared from rats pre-treated with aminoglutethimide. Addition of aminoglutethimide to cholesterol depleted cytochrome $P450_{scc}$, Soret absorption maximum 416 nm, results in the formation of a cytochrome $P450_{scc}$-aminoglutethimide complex, Soret absorption maximum 422 nm. Dialysis or gel filtration of the cytochrome $P450_{scc}$-amino-

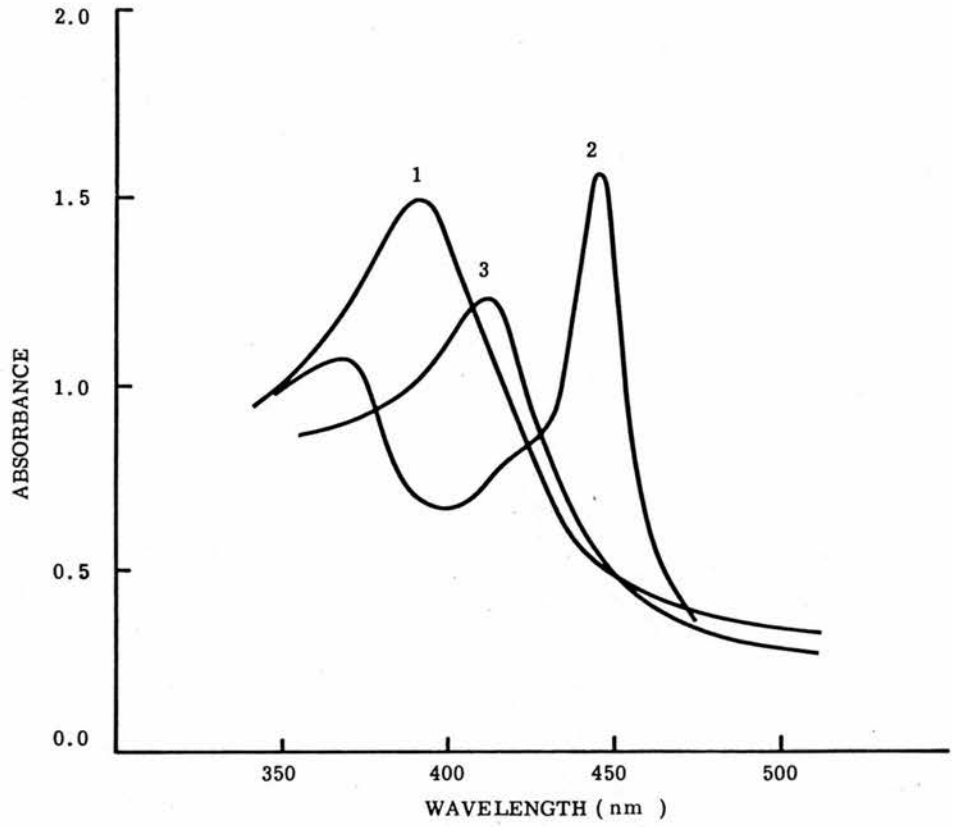


Fig. 3.5. DEGRADATION OF CYTOCHROME $P450_{scc}$-CHOLESTEROL COMPLEX BY POTASSIUM SUPEROXIDE

A 5 µl solution of potassium superoxide, stock solution 50 mM in dimethylsulphoxide, was added to a 2.5 ml solution of cytochrome $P450_{scc}$-cholesterol with the immediate formation of a haemoprotein having a Soret absorption at 412 nm. This degradation product was reduced by sodium dithionite but failed to bind carbon monoxide. The cytochrome $P450_{scc}$ concentration was 12.25 nmoles/ml with cholesterol 100 nmoles/ml and suspended in 10 mM potassium phosphate buffer pH 7.4.

curve 1 - ferric cytochrome $P450_{scc}$-cholesterol complex;

curve 2 - reduced carbon monoxide absolute spectrum of cytochrome $P450_{scc}$;

curve 3 - haemoprotein generated by addition of potassium superoxide to cytochrome $P450_{scc}$-cholesterol complex.

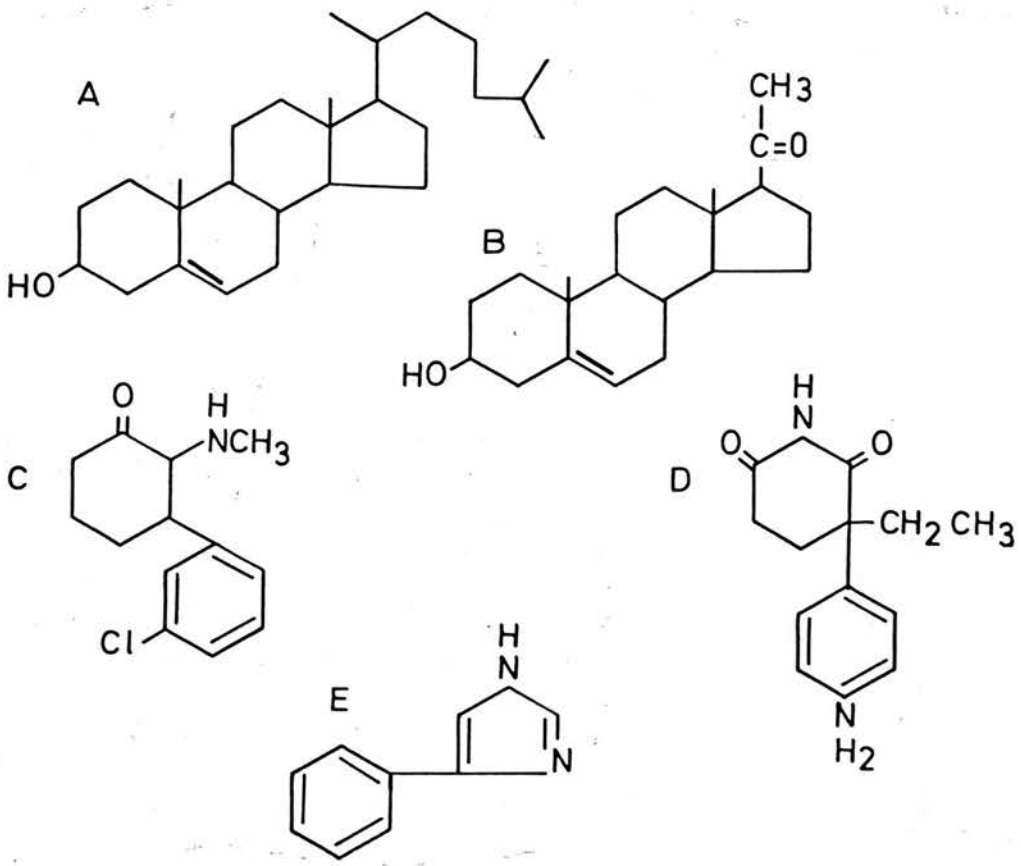
glutethimide complex returned the Soret absorption maximum to 416 nm and this is interpreted as indicative of removal of the aminoglutethimide ligand to the haemoprotein (Fig. 3.6).

4-Phenylimidazole forms a distinct spectral complex with cytochrome $P450_{CAM}$ (Gunsalus et al. (1972)) and in a similar manner to aminoglutethimide, dialysis or gel filtration of the cytochrome $P450_{scc}$-4-phenylimidazole complex results in reformation of the 416 nm Soret absorption band characteristic of the low spin substrate depleted cytochrome $P450_{scc}$.

Imidazole induced a spectral change in cytochrome $P450_{scc}$ similar to that of 4-phenylimidazole but with a lower affinity as measured by the spectral dissociation constant (Table 3.5).

Ketamine and pregnenolone induce inverse Type I spectral changes with 105,000 g supernatant fraction of a butanol-acetone powder extract. The inverse Type I spectral change is small, less than 10% of the total cytochrome P450 based on an extinction coefficient of 110 $mM^{-1}cm^{-1}$ (Peterson (1971)) for this spectral change and interpreted as ligand binding to that fraction of cytochrome $P450_{scc}$ in a high spin state. The inverse Type I spectral change induced by ketamine can be reversed by dialysis unlike that induced by pregnenolone. The recovery of cytochrome $P450_{scc}$ (Table 3.5) after ammonium sulphate fractionation in the presence of ketamine or pregnenolone suggests that the extent of binding may be greater than that observed by the inverse Type I spectral change. These ligands could be binding to low spin cytochrome $P450_{scc}$ without optical perturbation. In view of these doubts on the extent and nature of binding and subsequent reversibility, ketamine was not used as a protective ligand.

Aminoglutethimide and 4-phenylimidazole were the 'protective ligands' routinely used in view of their high affinity for cytochrome $P450_{scc}$ as



THE STRUCTURE OF PROTECTIVE LIGANDS USED DURING THE PURIFICATION OF CYTOCHROME $P450_{scc}$

A: cholesterol; B: pregnenolone; C: ketamine; D: amino-glutethimide and E: 4-phenylimidazole.

TABLE 3.5 Properties of ligands and stabilisation of cytochrome $P450_{scc}$ during ammonium sulphate fractionation of the 105,000 g supernatant of a butanol-acetone powder

The spectral dissociation constants were determined at the concentrations of cytochrome $P450_{scc}$ shown, on a low spin cytochrome $P450_{scc}$ preparation in 100 mM potassium phosphate buffer pH 7.4; specific content 4.4-6.1 nmoles cytochrome P450/mg protein. The inverse Type I spectral change induced by pregnenolone and ketamine were small; the spectral change induced represents less than 10% of the total cytochrome P450. The spectral changes induced by the ligands are as defined by Schenkman et al. (1967). The ligand concentration refers to the concentration added to cytochrome $P450_{scc}$ prior to ammonium sulphate fractionation. The reversibility of ligand binding is based on spectral shifts consequent upon dialysis or gel filtration.

Ligand	Cytochrome $P450_{scc}$ nmoles/ml	Spectral dissociation constant µM	Spectral change	Ligand concentration mM	%Recovery cytochrome $P450_{scc}$	Reversibility of ligand binding
Cholesterol	2.19	7.5	I	0.025	80%	-ve
Pregnenolone	2.5	5	small inverse I	0.025	83%	-ve
Ketamine	1.02	62	small inverse I	6.2	70%	+ve
4-Phenylimidazole	3.4	45	II	4.5	93%	+ve
Imidazole	3.4	50.10^3	II	-	-	-
Aminoglutethimide	3.4	3.5	II	0.35	95%	+ve
Control	3.4	-	-	-	33%	-

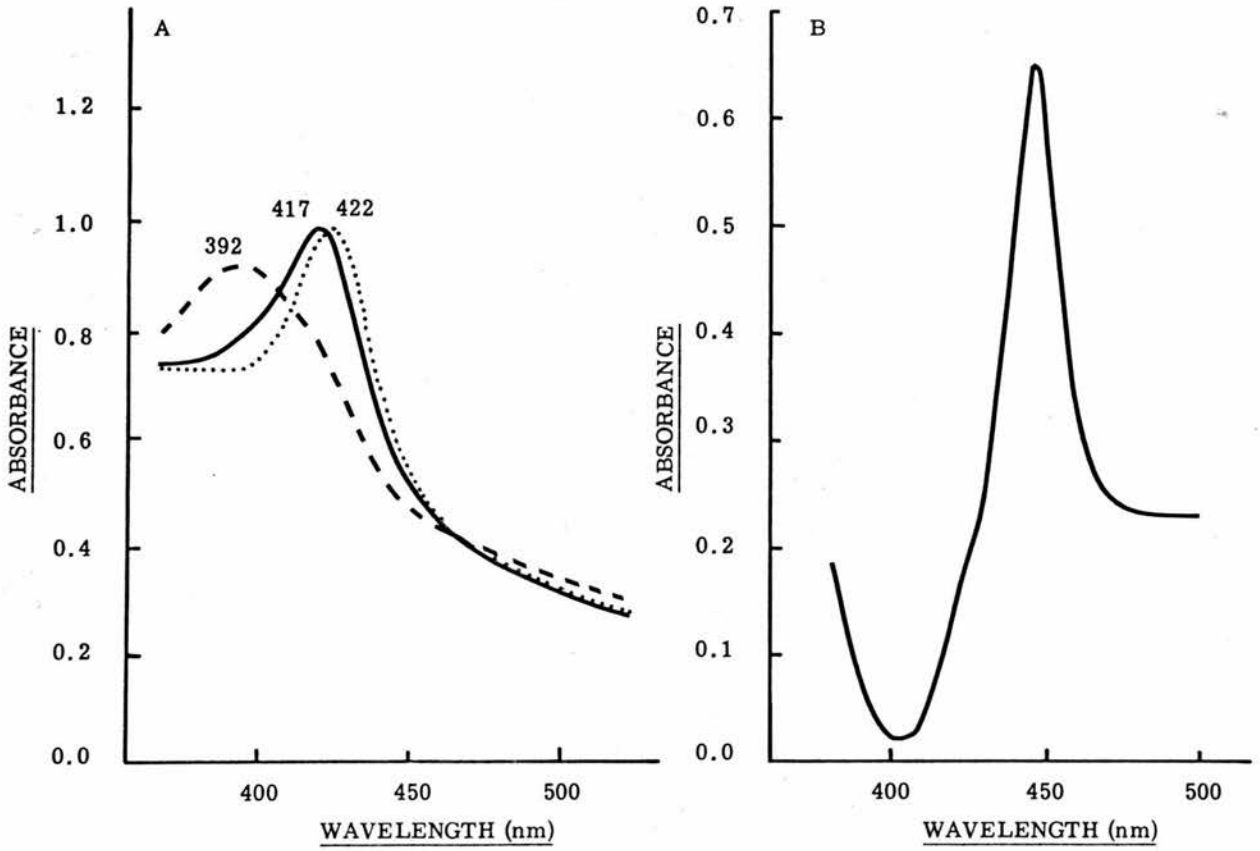


Fig. 3.6A. ABSOLUTE SPECTRA OF LIGAND INTERACTIONS WITH CYTOCHROME $P450_{scc}$

Cytochrome $P450_{scc}$, as isolated from the 105,000 g supernatant of a butanol-acetone powder, is a low spin cholesterol depleted haemoprotein _____ with a Soret absorption maximum at 417 nm. Addition of aminoglutethimide 500 μM results in the formation of a cytochrome $P450_{scc}$-aminoglutethimide complex ------- with a Soret absorption maximum at 422 nm; dialysis of this complex results in reformation of a spectral species with a Soret maximum of 417 nm. The addition of cholesterol 100 μm to low spin substrate depleted cytochrome $P450_{scc}$ results in the formation of a cytochrome $P450_{scc}$-cholesterol complex— — —with a Soret maximum at 392 nm; dialysis of this complex does not result in reversal of the spectral change. The cytochrome P450 concentration was 4.6 nmoles/ml in 10 mM potassium phosphate buffer pH 7.4.

B. THE REDUCED CARBON MONOXIDE DIFFERENCE SPECTRUM of low spin substrate depleted cytochrome $P450_{scc}$ with a Soret absorption maximum at 446 nm and a shoulder at 420 nm.

measured by the spectral dissociation constants, their reversibility of binding and the degree of protection afforded to the haemoprotein (Table 3.5).

The properties of ligands and recoveries of cytochrome $P450_{scc}$ after ammonium sulphate fractionation of the 105,000 g supernatant of a butanol acetone powder are given in Table 3.5. The results show that in the presence of exogenously added ligand the recovery of cytochrome $P450_{scc}$ is increased markedly compared to that of control without added ligand. The recovery of cytochrome $P450_{scc}$ is greater in the presence of 4-phenylimidazole or aminoglutethimide than in the presence of cholesterol or pregnenolone. However these groups of ligands cannot be directly compared as the molar ratios of ligand to cytochrome $P450_{scc}$ related to the apparent spectral dissociation constant of a ligand differ. For example the pregnenolone concentration given in Table 3.5 is fifty fold greater than the apparent spectral dissociation constant at that concentration of cytochrome $P450_{scc}$ whereas the similar ratio for aminoglutethimide is one hundred fold. The importance of defining the spectral dissociation constant in terms of cytochrome $P450_{scc}$ concentration will be discussed in Chapter 4 in relation to cholesterol binding. The effect of exogenously added ligands on cytochrome $P450_{scc}$ can be rationalised on the basis of knowledge of the endogenous axial ligands.

The first clue to the nature of the axial ligands of ferric cytochrome P450 was derived from EPR experiments. Miyake et al. (1968) demonstrated that ferric cytochrome P450 was predominantly a low spin ($S = \frac{1}{2}$) haemoprotein while Jefcoate and Gaylor (1969) and Blumberg et al. (1971) subsequently showed that the EPR spectra of low spin cytochrome P450 and cytochrome P420 (Stern et al. (1973)) were similar to those derived from the addition of mercaptoethanol or other alkyl

mercaptides to ferric haemoglobin or ferric myoglobin. These findings and various model studies with haemin thiol complexes (Bayer et al. (1969)) suggested that a necessary axial ligand for the iron in ferric cytochrome P450 is a thiol presumably a cysteine residue. Although one may infer the nature of the obligate axial ligands in the ferric protein from EPR data this is not possible for the ferrous state and synthetic models must be used.

A model compound was prepared by Stern and Peisach (1974) from haemin with the same optical properties in the Soret region as the carbon monoxide complex of the ferrous cytochrome P450. Obligate for the formation of this spectral species absorbing at 450 nm (EmM 93) and 555 nm (EmM 23) in a dimethylsulphoxide ethanol solvent was the presence of ferrous haem, carbon monoxide, thiol and a strong base. Decreasing the dielectric constant of the solvent which would be equivalent to increasing the hydrophobicity of the haem environment caused a small shift in the Soret absorption to the blue with no change in the extinction coefficient. The absence of thiol or strong base resulted in formation of a typical haem carbon monoxide complex with absorption at 413 nm (EmM 150), 535 (EmM 25), and 566 nm (EmM 30).

Nitrous oxide binds to cytochrome P450 producing optical spectra very different from nitrosyl haemoglobin or myoglobin (Ebel et al. (1975)). The optical spectra of nitrosyl cytochrome P450 can be duplicated by a model compound consisting of ferrous haem, sodium methyl mercaptide and nitrous oxide (Stern and Peisach (1976)) once more suggesting that thiolate anion is a ligand to the haem.

One physical study which set about to elucidate the chemical nature of the ligand counter to the sulphur in cytochrome P450 was that of Griffin et al. (1975). On the basis of proton nuclear magnetic resonance these authors suggested that water is the ligand trans to

the sulphur. However ligands other than water with exchangeable protons such as imidazole can relax water protons (Peisach (1973)). Further the electron paramagnetic resonance spectrum for low spin haem thiol complexes prepared in the absence of nitrogenous base has crystal field parameters sufficiently removed from those of cytochrome P450 to make it unlikely the possible participation of water as a haem ligand in the protein (Chevion et al. (1977)).

Analysis of a large number of model compounds and derivatives of haemoglobin, myoglobin as well as cytochrome P450 suggest that an imidazole is trans to the sulphur (Chevion et al. (1977)). From a comparison of crystal field parameters of haem thiol compounds it is suggested that exogenous ligands can exchange with endogenous haem ligands in the ferric cytochrome that is trans to the sulphur.

The ferrous protein model compound studies indicate that the haem ligand trans to the sulphur is displaced by either carbon monoxide (Stern and Peisach (1974)) or by nitrous oxide (Ebel et al. (1975)) as the optical spectrum of the carbon monoxide or nitrous oxide ligated forms could only be duplicated when an alkyl mercaptide was present.

Model reactions of exogenously added ligands to cytochrome $P450_{scc}$ have been proposed to account for the changes observed in spin state of the cytochrome (Griffin and Peterson (1972); Griffin et al. (1975) and Chevion et al. (1977)), and may be summarised as follows. In the low spin state, in the absence of exogenous ligands, the iron is six co-ordinated with sulphydryl and imidazole nitrogen groups from amino acid residues of the protein occupying the fifth and sixth ligand positions. In general substrates for cytochrome P450 dependent reactions are hydrophobic in character with few or only weakly ionisable functional groupings on the molecule. The ability to induce fundamental

changes in the spin state of cytochrome P450 by a substrate which does not directly interact with the iron of the haem has been rationalised in terms of indirect effects upon the protein moiety of cytochrome P450. Chevion et al. (1977) suggest that substrate interacts at a site proximal to the iron-porphyrin complex and perhaps by hydrophobic interaction with amino acid residues at this site causes a perturbation in protein structure which disrupts the iron-imidazole bond with a movement of the iron out of the plane of the porphyrin ring giving rise to the high spin state of the cytochrome and associated Type I optical spectral change.

Ligands which induce an inverse Type I spectral change are effecting a reversal of this process and strengthen the iron-imidazole bond forming a low spin state. The ligands with a nitrogenous functional grouping such as aminoglutethimide or 4-phenylimidazole are thought to directly ligand to the iron displacing the endogenous imidazole ligand and inducing a perturbed low spin state with a Type II optical spectral change.

The ligands used during the purification of cytochrome $P450_{scc}$ apparently must interact with the haem or protein components in different manners but having the same common property of maintaining the integrity of the haemoprotein. The hydrophobic nature of these protective ligands and their interaction with the protein component may be important in maintaining the structure of the haemoprotein.

Molecular Weight of Cytochrome $P450_{scc}$

The molecular weight of partially purified cytochrome $P450_{scc}$ was determined by gel filtration on Sepharose 4B. The column was calibrated with proteins of known molecular weight and the results plotted as the ratio of elution volume:void volume against the logarithm of molecular weight (Fig. 3.7). Cytochrome $P450_{scc}$, specific content 9.1 nmoles/mg

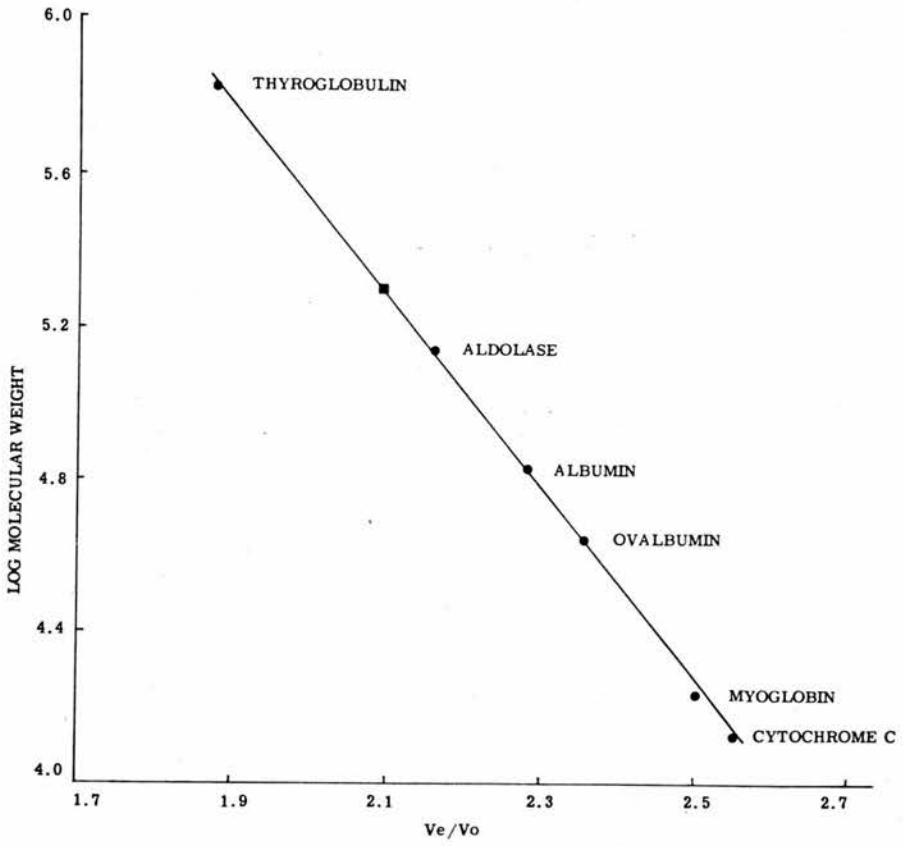


Fig. 3.7. DETERMINATION OF THE NATIVE MOLECULAR WEIGHT OF PARTIALLY PURIFIED CYTOCHROME $P450_{scc}$ BY GEL FILTRATION CHROMATOGRAPHY

Cytochrome $P450_{scc}$, specific content 9.1 nmoles/mg protein, and concentrated by ammonium sulphate precipitation was applied to a column of Sepharose 4B dimensions 45 x 1.5 cm pre-equilibrated with 10 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA, 100 mM potassium chloride and 500 μM aminoglutethimide and eluted with the same buffer at a flow rate of 20 ml/hr. Protein and cytochrome P450 eluted as a single peak. The results are plotted as the ratio of elution volume of cytochrome P450/void volume Ve/Vo against logarithm molecular weight. The column was calibrated with proteins of known molecular weight and the void volume determined with blue dextran.

Cytochrome $P450_{scc}$ ■

protein, was chromatographed on Sepharose 4B in the presence of 500 μM aminoglutethimide; protein and cytochrome $P450_{scc}$ eluted as a single peak with a molecular weight of 200,000.

The molecular weight of other partially purified preparations of cytochrome $P450_{scc}$ range from 60,000 (Horie and Watanabe (1975)) to 850,000 (Shikita and Hall (1973)). Within certain preparations of cytochrome $P450_{scc}$ there may be a single molecular cytochrome P450 species (Takemori et al. (1975)) or multiple molecular forms of this haemoprotein (Shikita and Hall (1973); Horie and Watanabe (1975)). Minimal molecular weight determinations of cytochrome $P450_{scc}$ give values of 46,000 (Takemori et al. (1975)), 53,000 (Shikita and Hall (1973)) and 60,000 (Wang and Kimura (1976)). These results suggest cytochrome $P450_{scc}$ aggregates during purification procedures.

Isoelectric Focusing of Cytochrome $P450_{scc}$

Analytical isoelectric focusing of cytochrome $P450_{scc}$ from the 105,000 g supernatant fraction of a butanol-acetone extract allows separation of multiple minor bands associated with peroxidase activity in the range pH 5.1-6.2 with the main bands at pH 5.3 and 5.4 (Fig. 3.8). Multiple bands with peroxidase activity reflect fundamentally different cytochrome P450s or simply charge variation dependent on degree of aggregation, bound cholate, trace phospholipid or the presence of degradation products with peroxidase activity such as cytochrome P420. The pI values previously determined for cytochrome P450 show a wide variation. Tilley et al. (1976) report a haem containing subunit of cytochrome $P450_{scc}$ with a pI of 7.0. The cytochrome $P450_{CAM}$ pI values are 4.55 (low spin) and 4.67 (high spin (Dus et al. (1970)) whereas a liver microsomal cytochrome P450 fraction LM2, homogeneous by polyacrylamide gel electrophoresis, gave four bands in the range pH 7-9 (Ingelman-Sundberg and Gustaffson (1977)). This range of pI

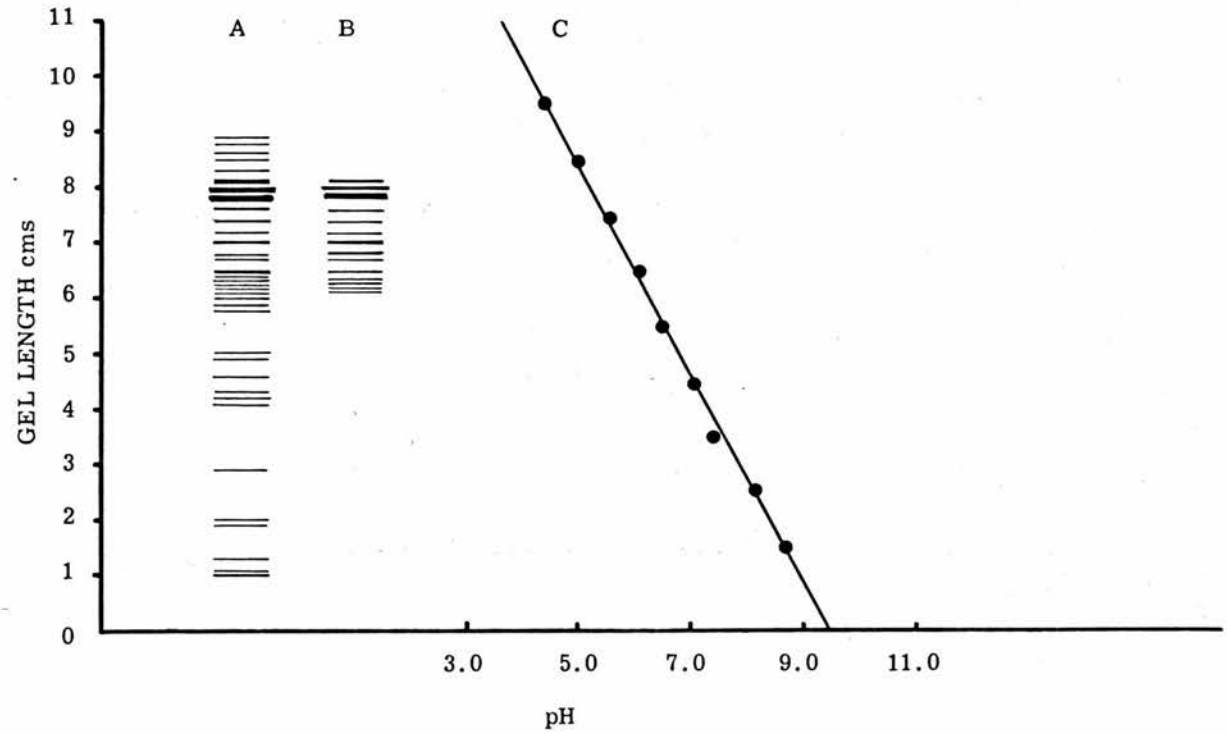


Fig. 3.8. ISOELECTRIC FOCUSSING ON POLYACRYLAMIDE GEL

Cytochrome $P450_{scc}$, specific content 5.1 nmoles/mg protein, was applied at neutral pH to a gel with a pre-established pH gradient and electrofocussing performed as described in Methods. A: represents protein bands observed after staining with Coomassie Blue; B: bands of peroxidative activity and C: the established pH gradient at the termination of the electrophoresis run.

values may simply represent variation in the methods employed, fundamental differences in the cytochrome P450s or the need for further purification to homogeneity and high specific content of the mammalian cytochrome.

Preparative isoelectric focusing in granulated gel using Ampholine range pH 4-6 resulted in precipitation of cytochrome $P450_{scc}$ at the zone of application with no migration of the cytochrome P450. Addition of 2% glycine or 1% Nonidet to the gel did not improve cytochrome P450 migration.

Further Purification of Cytochrome $P450_{scc}$

Cytochrome $P450_{CAM}$ is the terminal oxidase in Pseudomonas putida for the conversion of camphor to exo-5-hydroxycamphor (Katagiri et al. (1968)). This soluble cytochrome P450 has been purified and crystallised (Yu et al. (1974)) and has been used as a mechanistic model and guide for the purification of mammalian cytochrome P450. The resolution of membrane bound cytochrome P450 from mammalian sources and purification to high specific content with reasonable recovery has been less successful. Van der Hoeven et al. (1974) have purified liver microsomal cytochrome P450 to a high specific content, almost comparable to that of cytochrome $P450_{CAM}$, but yields are of the order of a few percent and the later kinetic experiments of these authors have been conducted with less pure cytochrome P450 (Peterson et al. (1977)). The situation is similar with the purification of cytochrome $P450_{scc}$ from adrenal cortex mitochondria. The preparations of Shikita and Hall (1973) and Wang and Kimura (1976) are of low specific content and have a low overall yield of cytochrome P450. The purification procedures of Takemori et al. (1975) and Horie and Watanabe (1975) give preparations with higher specific content, around 12 nmoles/mg protein but again with relatively low recovery. The need exists for

a preparation of mammalian cytochrome P450 of high specific content with a reasonable degree of recovery.

Further purification of cytochrome $P450_{scc}$ to a higher specific content has been attempted. The use of the chromatographic method of gel filtration has been investigated. The cytochrome $P450_{scc}$ fraction eluted from calcium phosphate gel as previously described was concentrated by ammonium sulphate precipitation to a final saturation of 50% and applied to a column of Biogel A 0.5 M, 50 2.5 cms, previously equilibrated with 10 mM potassium phosphate buffer containing 1 mM EDTA, 1 mM dithiothreitol and 0.5 mM aminoglutethimide and eluted with the same buffer solution at a rate of 25 mls/hr. The cytochrome $P450_{scc}$ eluted as a single peak with a Ve/Vo ratio of 1:1. The recoveries and specific content of cytochrome $P450_{scc}$ eluted are given in Table 3.6. The results indicate a loss of total cytochrome $P450_{scc}$ without concomitant formation of cytochrome P420 or the degradative product previously described. This loss of total cytochrome $P450_{scc}$ was associated with a decrease in specific content and coupled with the observation that rapid decolourisation of the cytochrome $P450_{scc}$ band occurred during the elution process has led to the conclusion that a degradative process resulting in a haemoprotein dissociation is probably involved. Exclusion of EDTA or dithiothreitol from the eluant made no significant difference to recovery of cytochrome $P450_{scc}$. Similarly increasing the dithiothreitol concentration to 10 mM or inclusion of glycerol to a 20% solution or a combination of both did not significantly increase recovery of cytochrome $P450_{scc}$. These agents have been useful in the partial reconversion of cytochrome P420 to cytochrome P450 but do not appear to reduce the extent of this haem-protein dissociation. Variation in ionic strength (0.01-0.1 μ); pH (6.8-7.8); temperature (4 and 20^{o}C); nature of eluting buffer (potassium phosphate or Tris

TABLE 3.6 Recovery of cytochrome $P450_{scc}$ after column chromatography

Cytochrome $P450_{scc}$ was applied to a column of Biogel A0.5M, 50 x 2.5 cms previously equilibrated with 10 mM potassium phosphate buffer pH 7.2 containing 1 mM EDTA, 1 mM dithiothreitol and 0.5 mM aminoglutethimide and eluted with the same buffer solution at a rate of 25 mls/hr. The cytochrome $P450_{scc}$ eluted as a single peak with a Ve/Vo ratio of 1:1. Cytochrome $P450_{scc}$ was applied in 10 mM potassium phosphate buffer pH 7.2 containing 1 mM EDTA, 1 mM dithiothreitol and 0.5 mM aminoglutethimide to a column of DEAE-cellulose 5 x 2 cms and then eluted with 100 mM potassium phosphate buffer pH 7.2 containing the same additives. The total nmoles and nmoles/mg protein refer in the table to nmoles cytochrome $P450_{scc}$ and nmoles cytochrome $P450_{scc}$/mg protein respectively.

	Cytochrome P450 Applied		Cytochrome P450 Recovered	
	Total nmoles	nmoles/mg protein	Total nmoles	nmoles/mg protein
Biogel A0.5M	48	10.1	29	8.5
DEAE-cellulose	62	9.8	31	6.5

hydrochloride) or the inclusion of n-propyl gallate (1 mM); the use of double glass distilled deionised water for buffer solutions; the use of argon saturated buffers; the exclusion of light during chromatography or the use of siliconised glassware or polypropylene columns did not reduce the extent of this degradation. The time of exposure of cytochrome $P450_{scc}$ to a chromatographic gel appears to be an important consideration and short column lengths with high flow rates within the limits of resolution of the gel results in less cytochrome loss. This was also observed on chromatographic gels where the cytochrome P450 was retained longer for the same flow characteristics such as Sepharose 4B or Biogel A1.5 M.

The use of ion exchange chromatography for further purification of cytochrome $P450_{scc}$ resulted in losses of the total cytochrome $P450_{scc}$ applied and also a decrease in specific content. In the example shown (Table 3.6) cytochrome $P450_{scc}$ was applied in 10 mM potassium phosphate buffer pH 7.2 containing 1 mM EDTA, 1 mM dithiothreitol and 0.5 mM aminoglutethimide to a column of DEAE-cellulose (5 cms x 2 cms) and then eluted with 100 mM potassium phosphate buffer pH 7.2 containing the same additives.

Hydrophobic interactions between cytochrome $P450_{scc}$ and aliphatic or aromatic substituents linked to Sepharose 4B have been used successfully by Takemori et al. (1975) as a method of purification of cytochrome $P450_{scc}$. In initial experiments using ω-aminohexyl- and ω-aminooctyl-Sepharose 4B no significant elution of cytochrome $P450_{scc}$ was obtained by simply increasing ionic strength and high concentrations of sodium cholate were required to elute cytochrome $P450_{scc}$. Hydrophobic interaction chromatography was not further explored at this time as it appeared counterproductive to aim for and attain a cholate depleted cytochrome $P450_{scc}$ preparation and then use detergent to elute from a

substituted Sepharose column.

The apparent loss of haem during the purification of mammalian cytochrome P450 has been a significant problem (Shikita and Hall (1973); Wang and Kimura (1976); Watanabe and Horie (1975); van der Hoeven et al. (1974)). However no detailed study has been made into the nature of this haem-protein dissociation observed particularly during chromatographic procedures. The degradation of cytochrome $P450_{scc}$ during column chromatography may be an oxidative process. The oxidative process could be indirect through peroxidation of associated lipid contaminants although inclusion of metal chelators or antioxidants did not reduce the extent of this degradation. Another possible oxidative attack could be a direct process through generation of an active oxygen species at the iron-porphyrin centre. The generation of a superoxy or a peroxy species would necessitate the initial reduction of the ferric iron prior to oxygen activation and one would have to postulate an internal reduction perhaps through an adjacent thiol residue. A more rigorous approach to the exclusion of molecular oxygen during column chromatography or the use of carbon monoxide as a protective ligand might be productive.

Summary

Cytochrome $P450_{scc}$, the terminal oxidase for the side chain cleavage of cholesterol to pregnenolone in bovine adrenal cortex mitochondria, was partially purified to a specific content of 10.2 nmoles cytochrome P450/mg protein with a recovery of 14%. The method involves iso-octane extraction of lyophilised mitochondria; ammonium sulphate fractionation in the presence of sodium cholate, butanol-acetone extraction, phosphate buffer extraction; a second ammonium sulphate fractionation, calcim phosphate gel adsorption and elution.

The cytochrome $P450_{scc}$ preparation has no detectable activity for the 11β-hydroxylation of deoxycorticosterone. Cholesterol: cytochrome $P450_{scc}$ molar ratios are reduced to 1:100 and the cytochrome has been isolated as a low spin haemoprotein. Adrenodoxin contamination in the preparation has been reduced to less than 1% on a molar basis to cytochrome $P450_{scc}$ and NADPH adrenodoxin reductase activity reduced to 4% of that in mitochondria. Sodium cholate, initially added to effect solubilisation, was reduced in the final preparation to a 3:1 molar ratio to cytochrome $P450_{scc}$. The molecular weight of cytochrome $P450_{scc}$, determined by gel filtration, was 200,000. The main bands of peroxidase activity observed using isoelectric focusing were at pH 5.3 and 5.4.

Further purification of cytochrome $P450_{scc}$ was limited by degradation of the cytochrome P450. A degradation product of cytochrome $P450_{scc}$ with a Soret absorption maximum at 412 nm which failed to bind carbon monoxide was observed. Ligands to cytochrome $P450_{scc}$, in particular 4-phenylimidazole and aminoglutethimide, were found to inhibit the degradation process.

CHAPTER 4

SPECTRAL INTERACTIONS OF CHOLESTEROL AND CYTOCHROME $P450_{SCC}$ FROM BOVINE ADRENAL CORTEX MITOCHONDRIA

INTRODUCTION

Cytochrome $P450_{scc}$ has been partially purified from bovine adrenal cortex mitochondria and isolated as a cholesterol depleted, low spin haemoprotein. This chapter describes the re-association of cholesterol with this partially purified preparation of cytochrome $P450_{scc}$ and factors which influence this reaction.

Limited amounts of cholesterol are available within adrenal mitochondria for use as substrate in the sterol side chain cleavage reaction (Simpson et al. (1971)). Elevation of the plasma ACTH increases the proportions of cholesterol in these mitochondria bound to cytochrome $P450_{scc}$ (Jefcoate et al. (1973)). Cycloheximide administration to rats tends to block the effects of subsequent ACTH administration and decreases pregnenolone production by adrenal cortex mitochondria but does not prevent the mitochondrial accumulation of cholesterol (Mahaffee et al. (1974); Arthur et al. (1976)). Jefcoate et al. (1974) implied that the cycloheximide sensitive process was dependent on the nature of the sterol substrate since the adrenal metabolism of 25-hydroxycholesterol was insensitive to the action of cycloheximide. Mason et al. (1978a) showed that analogues of cholesterol with non-polar alkyl side chains like cholesterol were sensitive to the cycloheximide pretreatment whereas the metabolism of cholesterol derivatives with polar side chains was insensitive. This implies that the cycloheximide sensitive factor or labile protein factor (Garren et al. (1965)) appears to be related to the transfer of non-polar sterols within the mitochondria to the oxygenase enzymes.

TABLE 4.1 Properties of cytochrome $P450_{scc}$ preparation

NADPH adrenodoxin reductase activity was measured by the method of Omura et al. (1964) and expressed as nmoles 2,6-dichlorophenolindophenol reduced/min/nmole cytochrome P450.

Absorption maxima	416, 535 and 568 nm
Specific content	6.3-7.8 nmoles cytochrome P450/mg prote
Cytochrome P450: cholesterol	1:0.01 molar ratio
Cytochrome P450: adrenodoxin	1:0.01 molar ratio
Cytochrome P450: phospholipid	1:3-6 molar ratio
Cytochrome P450: cholate	1:3 molar ratio
NADPH adrenodoxin reductase activity	less than 2% mitochondrial activity
Molecular weight	200,000
Enzymatic activity	Cholesterol side chain cleavage, no detectable 11β hydroxylase activity

RESULTS

Absolute Spectra

Cytochrome $P450_{scc}$ was partially purified from adrenal cortex mitochondria as previously described. The specific content of cytochrome $P450_{scc}$ used was 6.3-7.8 nmoles cytochrome P450/mg protein. Cytochrome $P450_{scc}$, as prepared, is a low spin substrate depleted haemoprotein with a cholesterol content less than 1% on a molar basis to cytochrome P450. The properties of the cytochrome $P450_{scc}$ preparation are shown in Table 4.1.

The absorption maxima of low spin cholesterol depleted cytochrome $P450_{scc}$ are 416, 535 and 568 nm. Addition of cholesterol to low spin substrate depleted cytochrome $P450_{scc}$ results in a decrease in absorbance at 416 and 568 nm and an increase in absorbance at 392 and 648 nm with isosbestic points at 406, 457, 533 and 588 nm (Fig. 4.1) to produce a spectral species which is identical to that prior to butanol-acetone extraction. The molar ratio cholesterol:cytochrome $P450_{scc}$ prior to butanol-acetone extraction is about 9:1 with cytochrome $P450_{scc}$ in a predominantly high spin state and after butanol-acetone extraction the ratio is 0.01:1 with cytochrome $P450_{scc}$ predominantly in a low spin form.

Spectral Dissociation Constants

Addition of non-saturating amounts of cholesterol to low spin, substrate depleted cytochrome $P450_{scc}$ results, at equilibrium, in an increasing proportion of cytochrome $P450_{scc}$ in a high spin state as the cholesterol concentration increases to saturation (Fig. 4.1). If these optical changes are monitored as difference spectra, a Type I optical change (Schenkman et al. (1967)) is observed with absorbance maximum at 385 nm and minima at 420 and 570 nm (Fig. 4.2). A plot of absorbance change (absorbance 385 nm minus absorbance 420 nm)

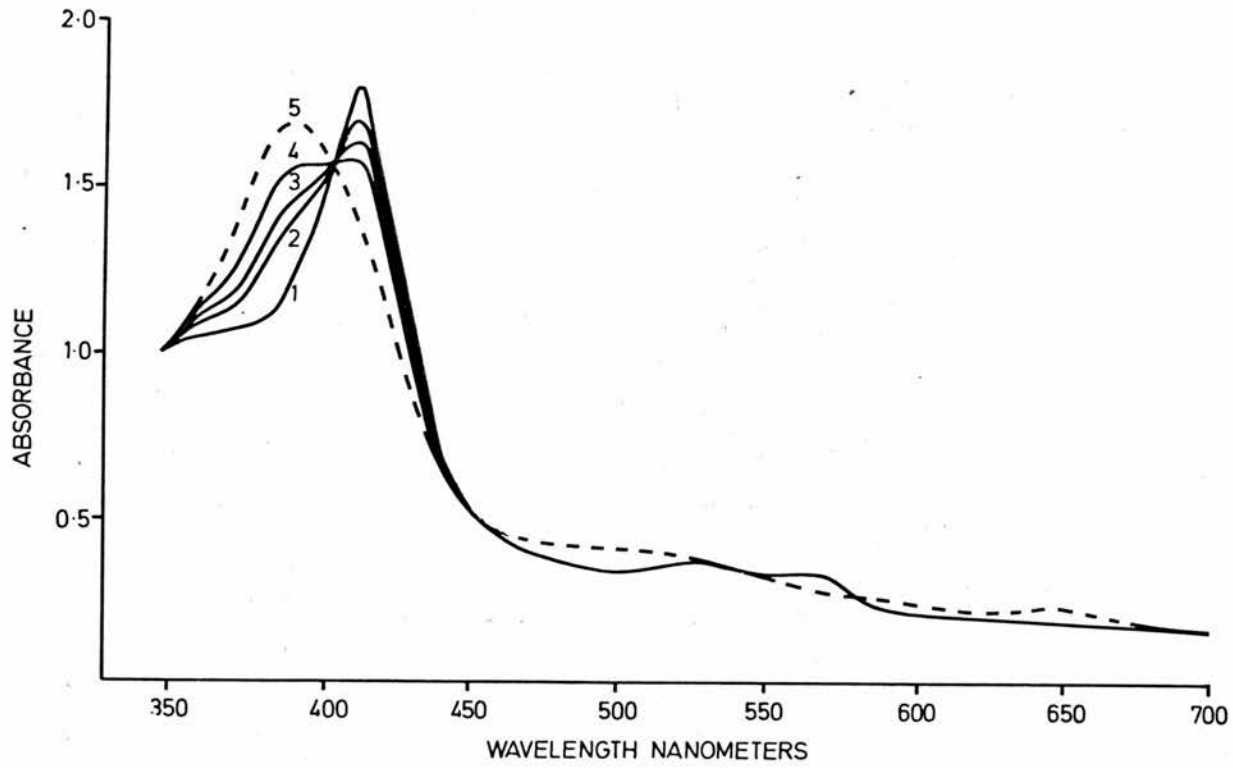


Fig. 4.1. ABSOLUTE SPECTRAL CHANGES AT EQUILIBRIUM ON ADDITION OF CHOLESTEROL TO LOW SPIN CYTOCHROME $P450_{scc}$

Cholesterol, stock solution 2 mM in propylene glycol, was added to a 1 ml spectrophotometer cuvette containing low spin cytochrome $P450_{scc}$. The cytochrome $P450_{scc}$ concentration was 17.2 nmoles/ml in 10 mM potassium phosphate buffer pH 7.4. 1 mM EDTA and spectra were recorded after incubation for 15 hrs at 20°C. The concentrations of added cholesterol were 1, zero; 2, 5 μM; 3, 13.8 μM; 4, 17 μM and 5, 50 μM.

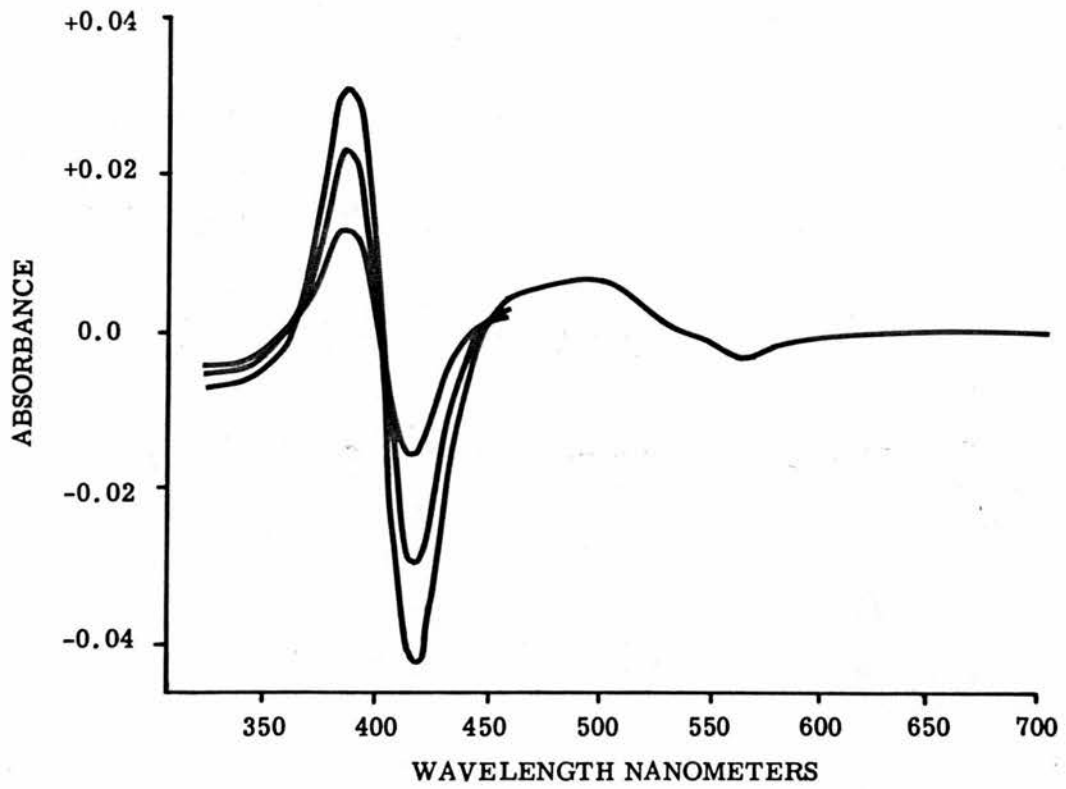


Fig. 4.2. DIFFERENCE SPECTRA OBSERVED ON ADDITION OF CHOLESTEROL TO LOW SPIN CYTOCHROME P450

Cholesterol, stock solution 2 mM in propylene glycol, was added to a 1 ml spectrophotometer cuvette containing low spin cytochrome $P450_{scc}$. The cytochrome $P450_{scc}$ concentration was 0.75 nmoles/ml in 10 mM potassium phosphate buffer pH 7.4 with 1 mM EDTA and after cholesterol addition the samples were incubated for 15 hrs at 20°C prior to spectral observation. The difference spectra of samples were recorded against a control containing low spin cytochrome $P450_{scc}$ with no added cholesterol but with addition of an appropriate concentration of propylene glycol. The added cholesterol concentrations were 1, 0.5 μM; 2, 1.5 μM; and 3, 2.0 μM.

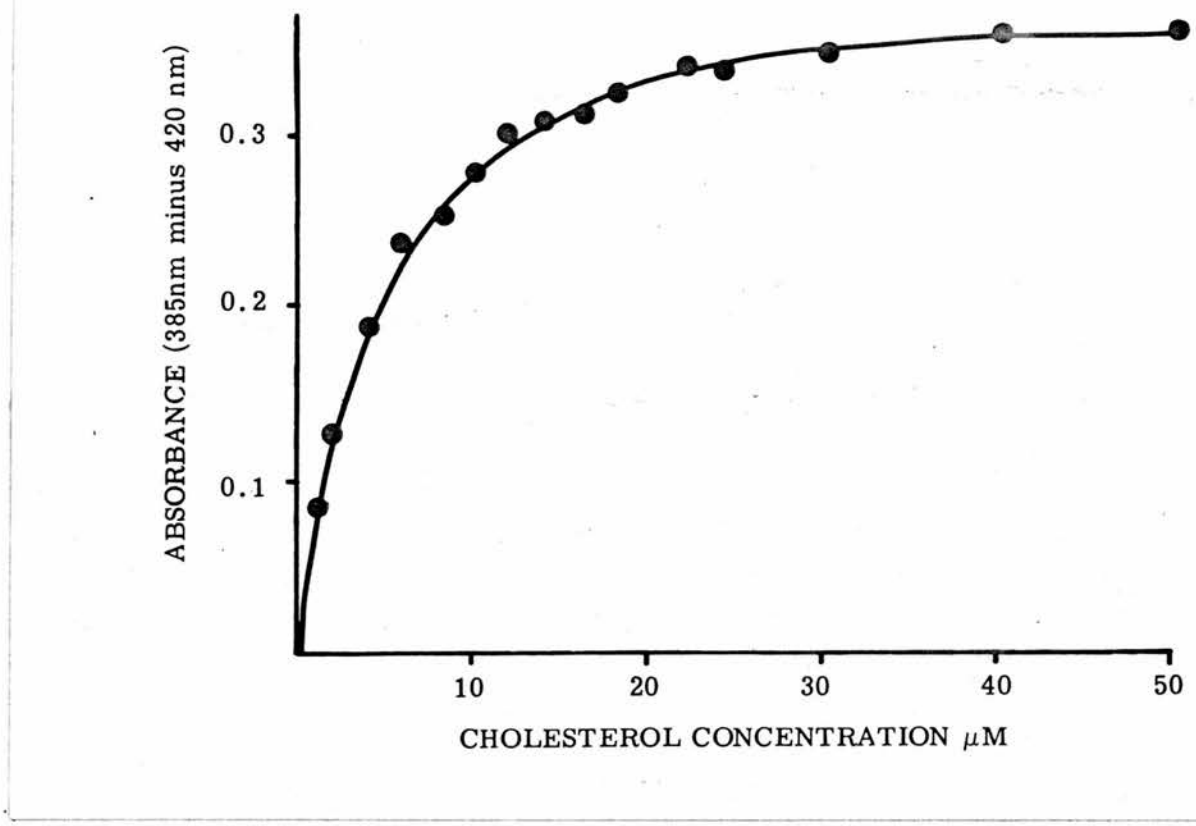


Fig. 4.3. RELATIONSHIP OF ABSORBANCE CHANGE (385-420 nm) OF CYTOCHROME $P450_{scc}$ TO CHOLESTEROL CONCENTRATION ADDED

Cholesterol, stock solution 2 mM in propylene glycol, was added to a low spin cytochrome $P450_{scc}$ preparation containing 3.6 nmoles cytochrome P450/ml in 10 mM potassium phosphate pH 7.4, and allowed to incubate at 20°C for 15 hrs prior to spectral measurements. The samples containing cholesterol were read against a control containing no added cholesterol and the absorbance difference 385 minus 420 nm plotted against cholesterol concentration.

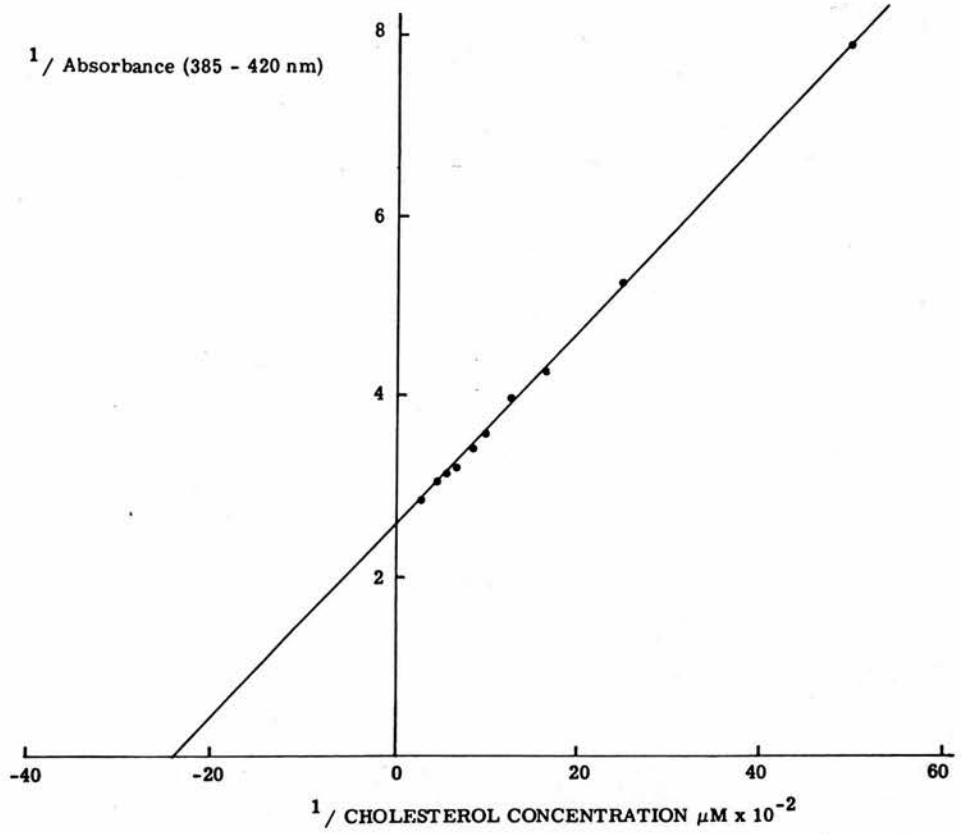


Fig. 4.4. DOUBLE RECIPROCAL PLOT OF ABSORBANCE CHANGE AGAINST CHOLESTEROL CONCENTRATION

The data is taken from Fig. 4.3 and allows calculation of the spectral dissociation constant Ks and the maximal absorbance change induced by the ligand.

against cholesterol concentration (Fig. 4.3) shows an increase in absorbance change with increase in cholesterol concentration towards saturation. A plot of the reciprocal of absorbance change against the reciprocal of the cholesterol concentration allows calculation of the spectral dissociation constant Ks and the maximal absorbance change induced by the ligand (Fig. 4.4).

Equilibrium Constant

A plot of the spectral dissociation constant Ks of cholesterol against concentration of cytochrome $P450_{scc}$ is shown in Fig. 4.5 at two different potassium phosphate buffer concentrations. The spectral dissociation constant varies with the enzyme concentration and these results are expected if the concentration of free cholesterol during the titration is not much greater than the concentration of bound cholesterol. The observed changes in the absorption spectrum of cytochrome $P450_{scc}$ on addition of cholesterol can be used to calculate the equilibrium constant of the reaction:

$$E + \text{cholesterol} \rightleftharpoons E\text{-cholesterol}$$

using the method developed by Peterson (1971) for camphor binding to cytochrome $P450_{cam}$. The assumptions made for the calculations are:

a) the concentration of free cytochrome $P450_{scc}$ (E) is equal to the total concentration of cytochrome $P450_{scc}$ (E_t) minus the concentration of the cholesterol complex of cytochrome $P450_{scc}$ (E-cholesterol);

b) the concentration of the cholesterol complex was calculated from the absorbance of the Type I change using an extinction coefficient of 110 $mM^{-1}cm^{-1}$ (Peterson (1971));

c) the concentration of free cholesterol is equal to the total concentration of cholesterol added minus cholesterol bound to cytochrome $P450_{scc}$.

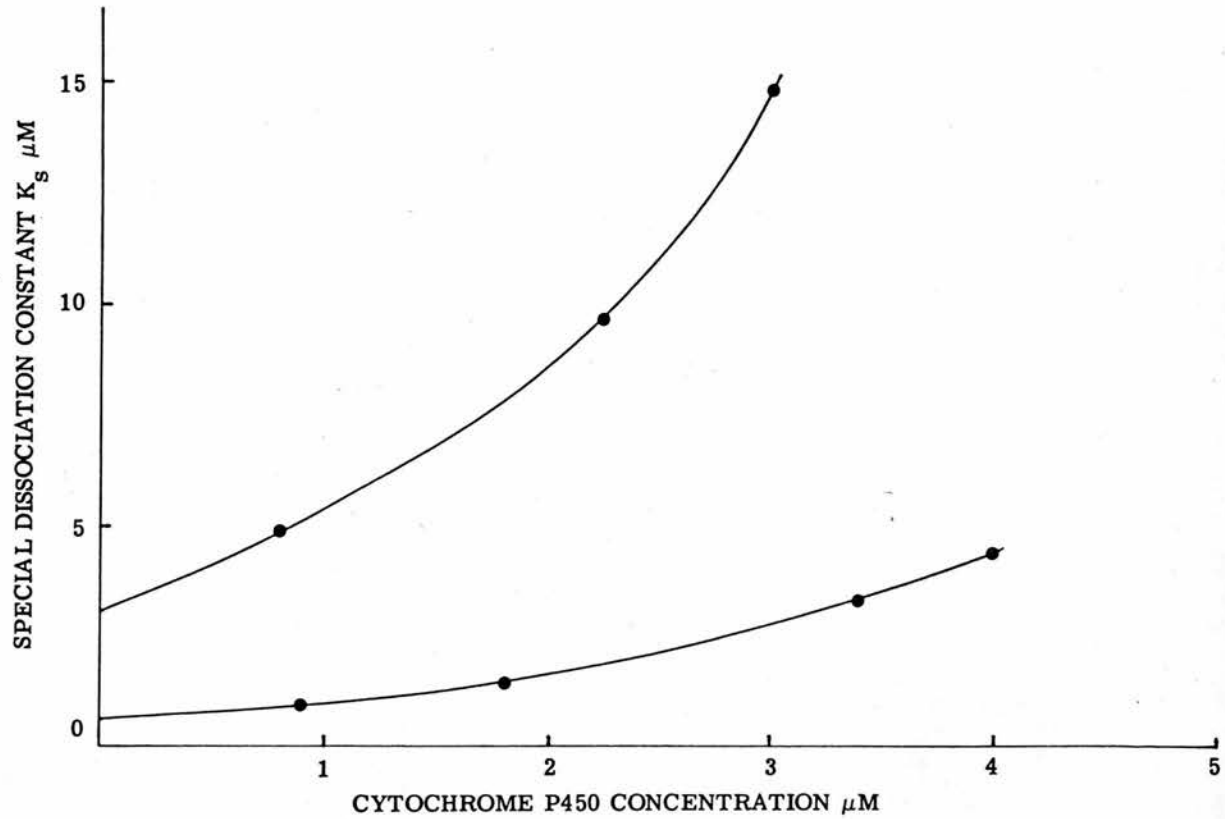


Fig. 4.5. THE RELATIONSHIP BETWEEN THE SPECTRAL DISSOCIATION CONSTANT Ks OF CHOLESTEROL AND CYTOCHROME P450$_{scc}$ CONCENTRATION

The specrral dissociation constants were determined from a reciprocal plot of absorbance change (385-420 nm) against cholesterol concentration for a particular cytochrome P450 concentration. The lower and upper curves represent spectral dissociation constants determined in 10 mM and 100 mM potassium phosphate buffer pH 7.4 at 20^{o}C respectively.

The equilibrium constant

$$K_{equil} = \frac{(E\text{-cholesterol})}{(E).\ (Cholesterol)} = 0.43 \pm 0.03 \times 10^{6} M^{-1}$$

This value is valid for cytochrome $P450_{scc}$ in 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA at 20°C.

Effect of Potassium Chloride on Spin States at Equilibrium

The effect of potassium chloride concentration on the Type I spectral change induced with a non-saturating (lower curve) and saturating (upper curve) amount of cholesterol is shown in Fig. 4.6. Potassium chloride was added to 1 ml aliquots of low spin cytochrome $P450_{scc}$, concentration 2.75 nmoles/ml, in 20 mM Tris hydrochloride buffer at 20°C. Cholesterol concentrations were 4 μM (lower curve) and 100 μM (upper curve). The control sample of cytochrome $P450_{scc}$ on the lower curve with added cholesterol but no potassium chloride has a $K_{equil} = 0.44 \times 10^{6}\ M^{-1}$ and 47% of the cytochrome $P450_{scc}$ was in a high spin state; with 100 mM potassium chloride added the $K_{equil} = 0.08 \times 10^{6}\ M^{-1}$ and 21% of the cytochrome $P450_{scc}$ was in a high spin state. The control sample, the upper curve, was 85% high spin compared to 60% high spin in the presence of 100 mM potassium chloride. Concentrations of potassium chloride greater than 100 mM resulted in formation of cytochrome P420 and an increase in turbidity as measured by the absorbance at 457 nm.

The data from Fig. 4.6 was plotted as absorbance change (absorbance 385 nm minus absorbance 420 nm) potassium chloride concentration against absorbance change (Fig. 4.7). A biphasic plot was obtained on data from the lower curve (non-saturating cholesterol) with K_I values for potassium chloride of 5 mM and 77 mM, calculated from the slope of the plots $-\frac{I}{K}$. A linear plot was obtained from the upper curve (saturating cholesterol) with a K_I = 104 mM. These results suggest that potassium chloride appears tohave two effects on inhibition and

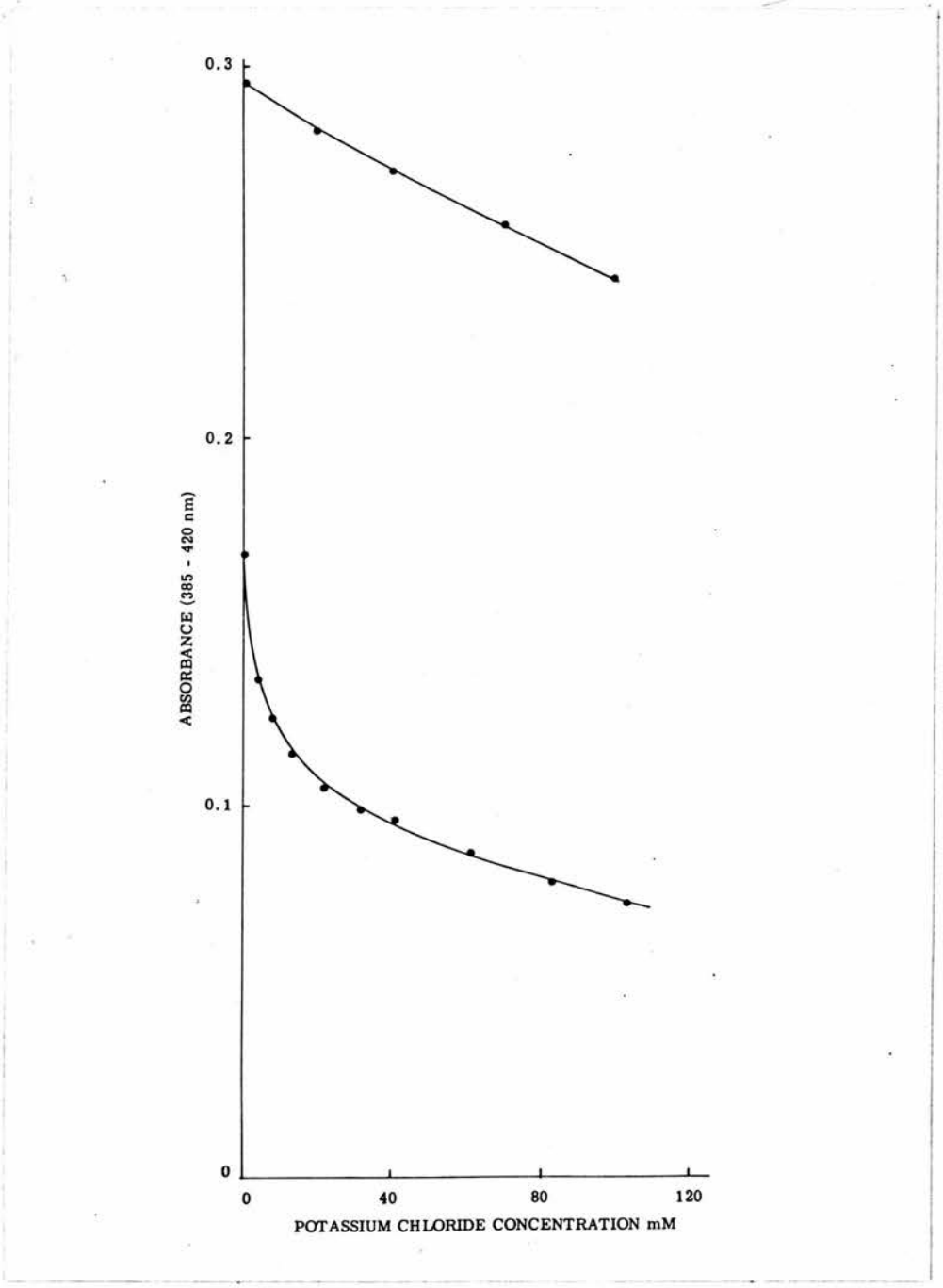


Fig. 4.6. THE EFFECT OF POTASSIUM CHLORIDE ON THE CHOLESTEROL INDUCED LOW TO HIGH SPIN STATE TRANSITIONS OF CYTOCHROME $P450_{scc}$ AT EQUILIBRIUM

Potassium chloride was added to 1 ml aliquots of low spin cytochrome $P450_{scc}$ concentration 2.75 nmoles cytochrome P450/ml, in 20 mM Tris hydrochloride buffer pH 7.4 at 20°C. Cholesterol, stock solution 2 mM in propylene glycol, was added to each aliquot to a concentration of 4 μM (lower curve) and to 100 μM (upper curve). The samples were incubated at 20°C for 15 hrs and the Type 1 absorbance changes recorded against a control with no cholesterol or potassium chloride added.

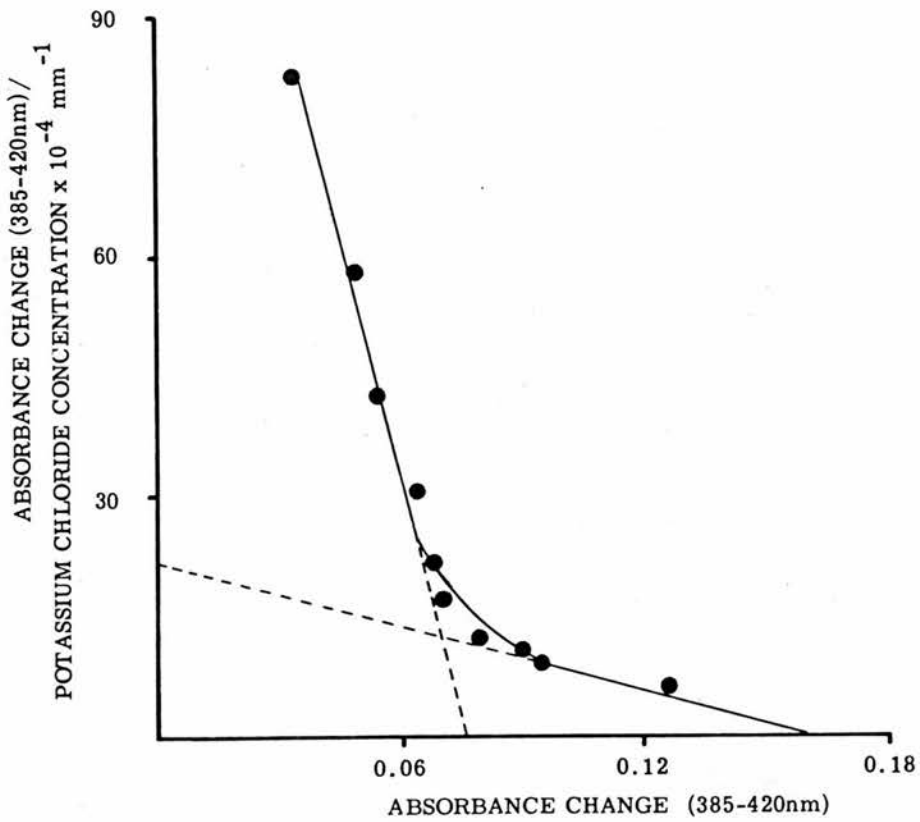


Fig. 4.7. A PLOT OF ABSORBANCE CHANGE (385-420 nm) POTASSIUM CHLORIDE CONCENTRATION AGAINST ABSORBANCE CHANGE (385-420 nm)

The data was taken from the lower curve (non-saturating cholesterol concentration) Fig. 4.6 and plotted as above gives slopes = $-\frac{I}{KI}$ and maximal absorbance changes from extrapolation to the x axis. The biphasic plot has K Inhibitory (KI) values for potassium chloride of 5 mM and 77 mM on cholesterol induced low to high spin state transition of cytochrome $P450_{scc}$ at equilibrium.

the inhibitory effect with the lower K_I value is antagonised by cholesterol.

Rate of Cholesterol Induced Low to High Spin State Transition of Cytochrome $P450_{scc}$

The addition of a saturating amount of cholesterol at time zero to low spin substrate depleted cytochrome $P450_{scc}$ followed by observations on the absorbance change (absorbance 385 nm minus absorbance 420 nm) with time results in a plot as shown in Fig. 4.8. The results were subsequently plotted as a first order reaction with respect to (Af-At); the difference between the final absorbance change Af and that at time t, At (Fig. 4.9). The results were plotted as a first order reaction since under these conditions not all of the cytochrome $P450_{scc}$ is converted to the enzyme-cholesterol complex; (Af-At) is not directly proportional to the total unbound cytochrome P450 at time t but only that part which remains to be bound to cholesterol at equilibrium. The first order rate constant was calculated from Fig. 4.9 using the relationship slope = $\frac{k}{2.303}$ with a value for $k = 7.5 \times 10^{-4}$ sec^{-1}; valid for binding of cholesterol to low spin cytochrome $P450_{scc}$ in 10 mM potassium phosphate buffer pH 7.4 at $20^{o}C$.

Effect of Potassium Chloride on the Rate of Cholesterol Binding to Cytochrome $P450_{scc}$

The Type I spectral change induced by the addition of saturating amounts of cholesterol to low spin cytochrome $P450_{scc}$ were observed with time in the absence and presence of varying potassium chloride concentrations. The first order rate constants were calculated and the relationship to potassium chloride concentration shown in Fig. 4.10. The effect of potassium chloride is to decrease the first order rate constant of association of cholesterol with cytochrome $P450_{scc}$. The inhibitory effect of monovalent ions contrasts markedly with the

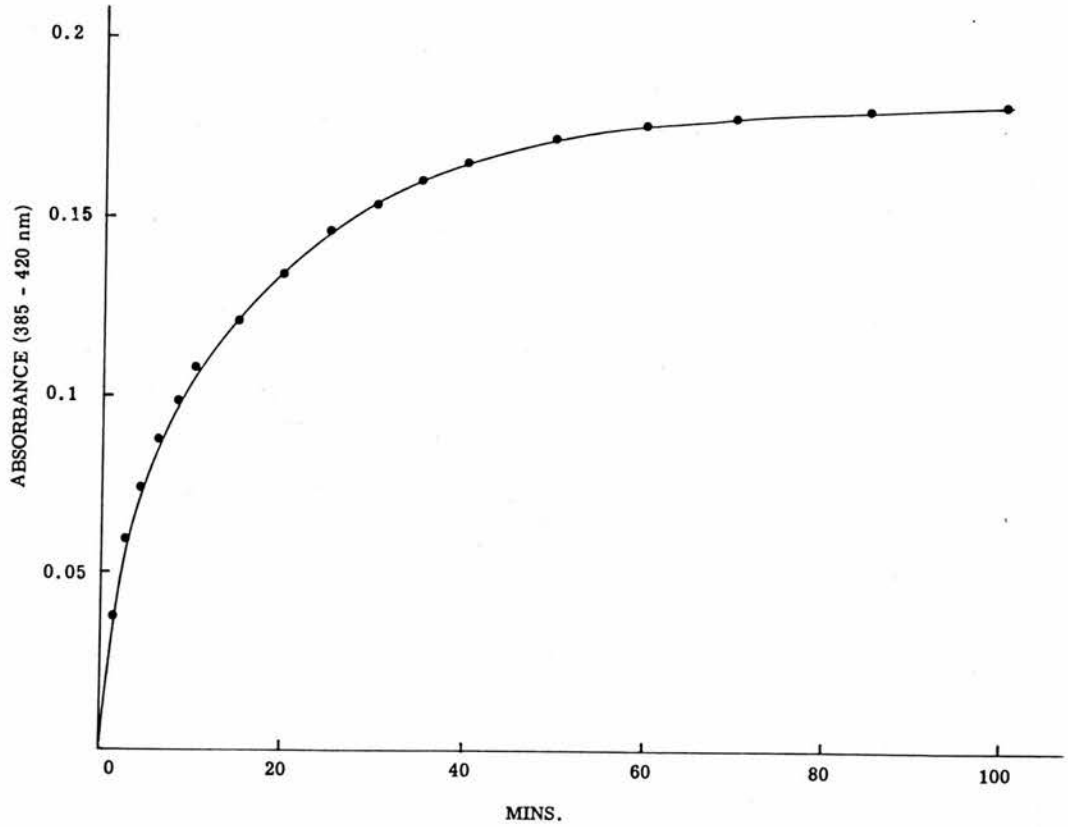


Fig. 4.8. A PLOT OF ABSORBANCE CHANGE (385-420 nm) ON ADDITION OF CHOLESTEROL TO LOW SPIN CYTOCHROME $P450_{scc}$ AGAINST TIME

Cholesterol 100 μM, stock solution 2 mM in propylene glycol, was added at time zero to low spin cytochrome $P450_{scc}$ in a 1 ml spectrophotometer cuvette. Cytochrome $P450_{scc}$ concentration was 1.7 nmoles/ml in 10 mM potassium phosphate buffer pH 7.4. The reaction temperature was 20°C and the Type 1 spectral change was followed with time.

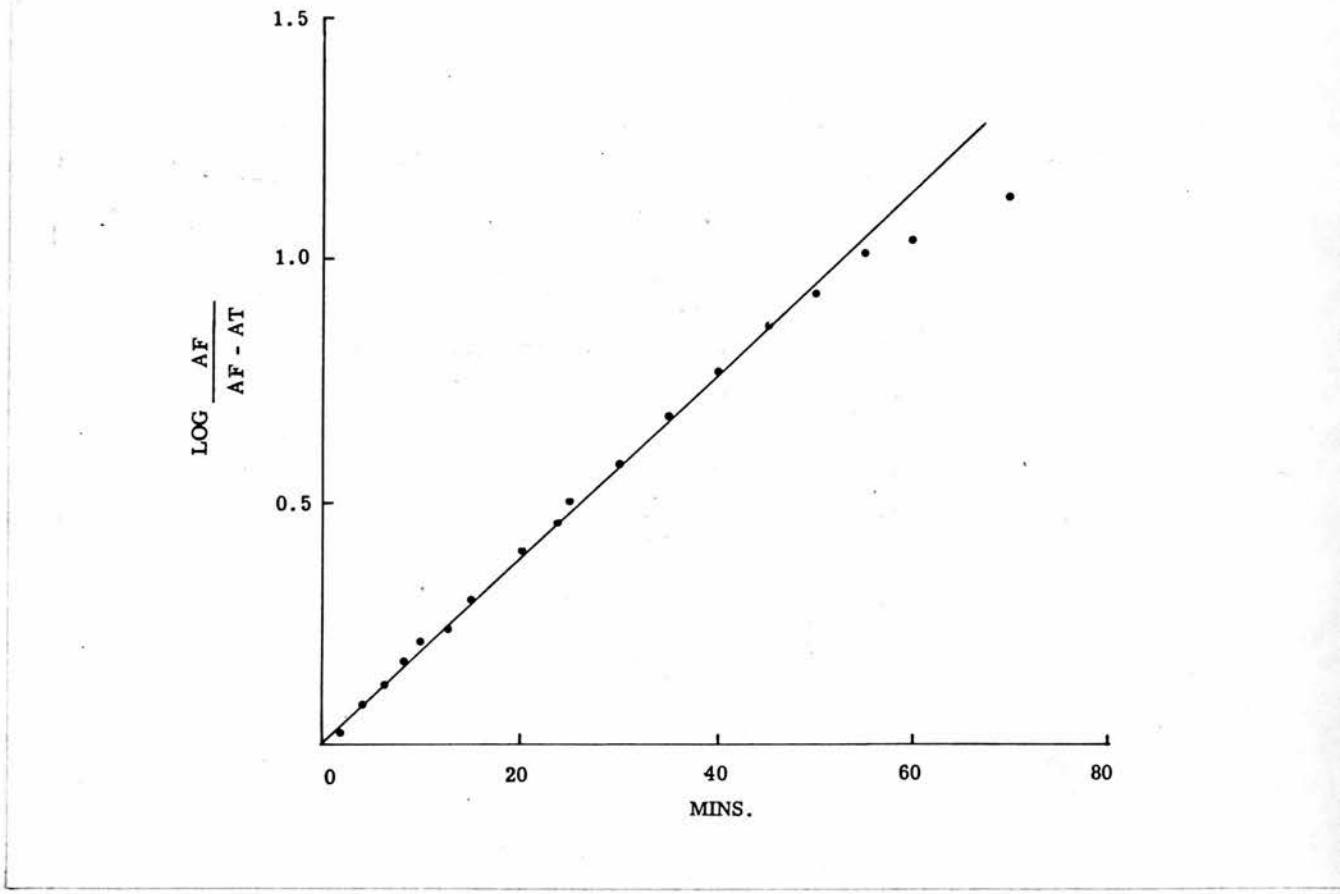


Fig. 4.9. A FIRST ORDER PLOT OF THE REACTION OF CHOLESTEROL WITH LOW SPIN CYTOCHROME $P450_{scc}$

The data is taken from Fig. 4.8 and plotted as a first order reaction with respect to (Af-At); the difference between final absorbance change Af and that at time t, At. From the relationship slope $\frac{k}{2.303}$; the first order rate constant was calculated $k = 7.5 \times 10^{-4} sec^{-1}$. First order kinetics were observed for 85% of the absorbance change.

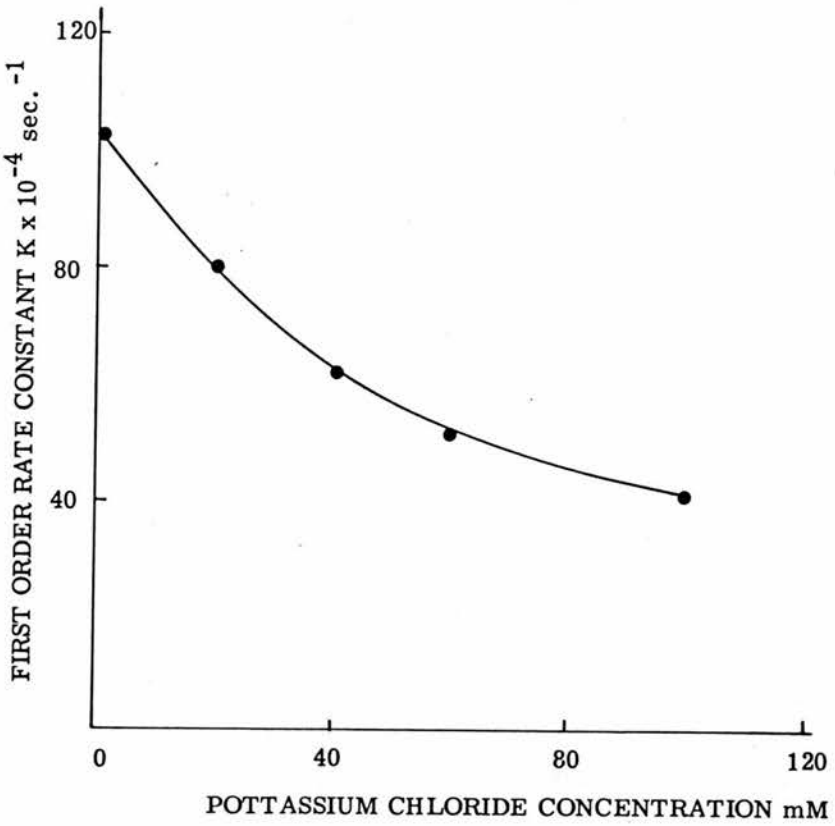


Fig. 4.10. THE EFFECT OF POTASSIUM CHLORIDE ON THE RATE OF CHOLESTEROL INDUCED LOW TO HIGH SPIN STATE CHANGE OF CYTOCHROME $P450_{scc}$

Cholesterol (final concentration 100 μM), stock solution 2 mM in propylene glycol, was added at time zero to a 1 ml spectrophotometer cuvette containing low spin cytochrome $P450_{scc}$ and the absorbance change (385-420 nm) recorded with time. The cytochrome $P450_{scc}$ concentration was 2.1 nmoles/ml in 50 mM Tris hydrochloride buffer pH 7.0 at the reaction temperature of 20°C. Potassium chloride was added 5 mins prior to the addition of cholesterol. The first order rate constants were calculated and the constants plotted against potassium chloride concentrations.

stimulatory pattern of the divalent ion, calcium.

Effect of Calcium Chloride on the Rate of Cholesterol Binding to Cytochrome $P450_{scc}$

The Type I spectral changes induced by the addition of saturating amounts of cholesterol to low spin cytochrome $P450_{scc}$ were observed with time in the presence and absence of calcium chloride (Fig. 4.11). The first order rate constants were calculated and the relationship to concentration of calcium chloride shown in Fig. 4.12. Maximal increase in the first order rate constant was noted at about 1 mM calcium chloride concentration. No increase in the first order rate constant of the association of cholesterol with low spin cytochrome $P450_{scc}$ was noted in the presence of magnesium or manganese chloride. A similar specificity for calcium ions was noted by Mason et al. (1978b) for the stimulation of pregnenolone production in intact mitochondria.

Effect of Temperature on the Rate of Cholesterol Binding to Cytochrome $P450_{scc}$

First order rate constants of cholesterol induced low to high spin state change were determined as previously described over the range 4-37°C. The influence of temperature on the reaction velocity can be expressed in the form of the Arrhenius equation, $k = Ae^{-E/RT}$ where k is the reaction rate, $e^{-E/RT}$ is the Boltzman factor, A is a constant and E is the activation energy of the reaction. The logarithmic form of the equation, $\log k - \log A = \frac{E}{2.303RT}$ requires a plot of log k against I/T to be linear with a gradient equal to E/2.303RT. A plot of the logarithm of the first order rate constants against the reciprocal of the absolute temperature is linear (Fig. 4.13) and the calculated activation energy E = 88 kJ $mole^{-1}$. The activation energy of the low to high spin state equilibrium of cytochrome $P450_{scc}$ in intact rat adrenal

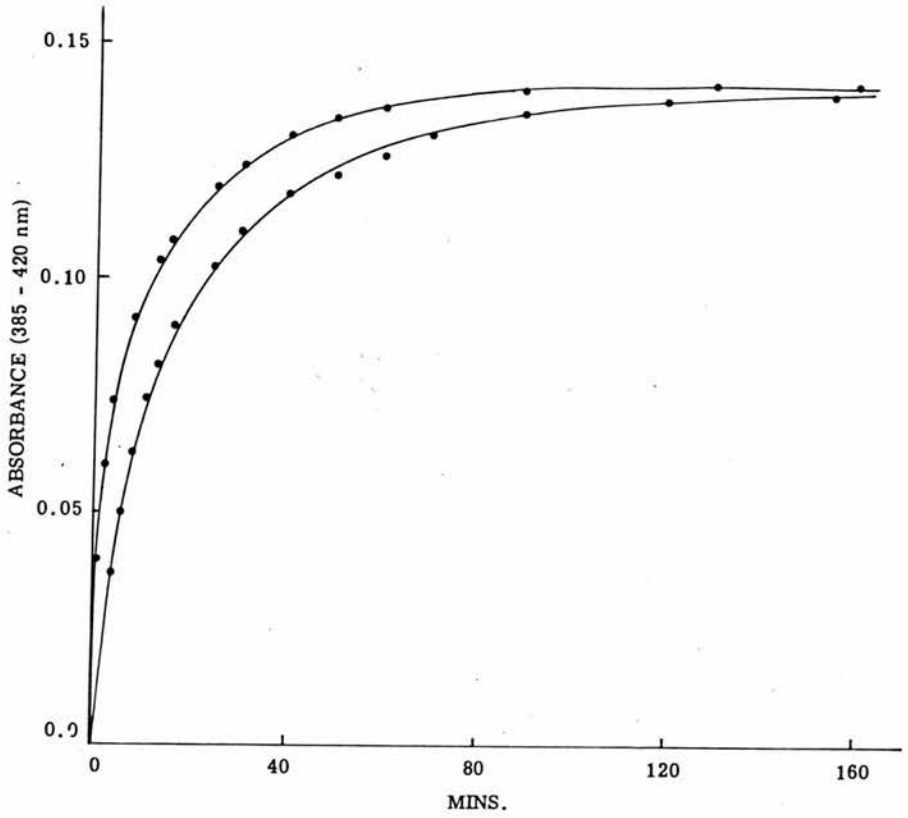


Fig. 4.11. THE EFFECT OF CALCIUM CHLORIDE ON THE RATE OF CHOLESTEROL INDUCED LOW TO HIGH SPIN STATE TRANSITION OF CYTOCHROME $P450_{scc}$

Cholesterol (final concentration 100 μM) stock solution 2 mM in propylene glycol, was added at time zero to a 1 ml spectrophotometer cuvette containing low spin cytochrome $P450_{scc}$ and the absorbance change (385-420 nm) recorded with time. The cytochrome $P450_{scc}$ concentration was 1.75 nmoles/ml in 50 mM Tris hydrochloride buffer pH 7.0 containing 100 mM potassium chloride. The reaction temperature was 20°C. Calcium chloride was added to a 1 mM concentration 5 mins prior to the addition of cholesterol and the consequent spectral changes with time represented by the upper curve. The control reaction without calcium chloride is represented by the spectral changes shown in the lower curve. The first order rate constants in the presence of calcium chloride was 11.4×10^{-4} and in the absence of calcium chloride 7.0×10^{-4}.

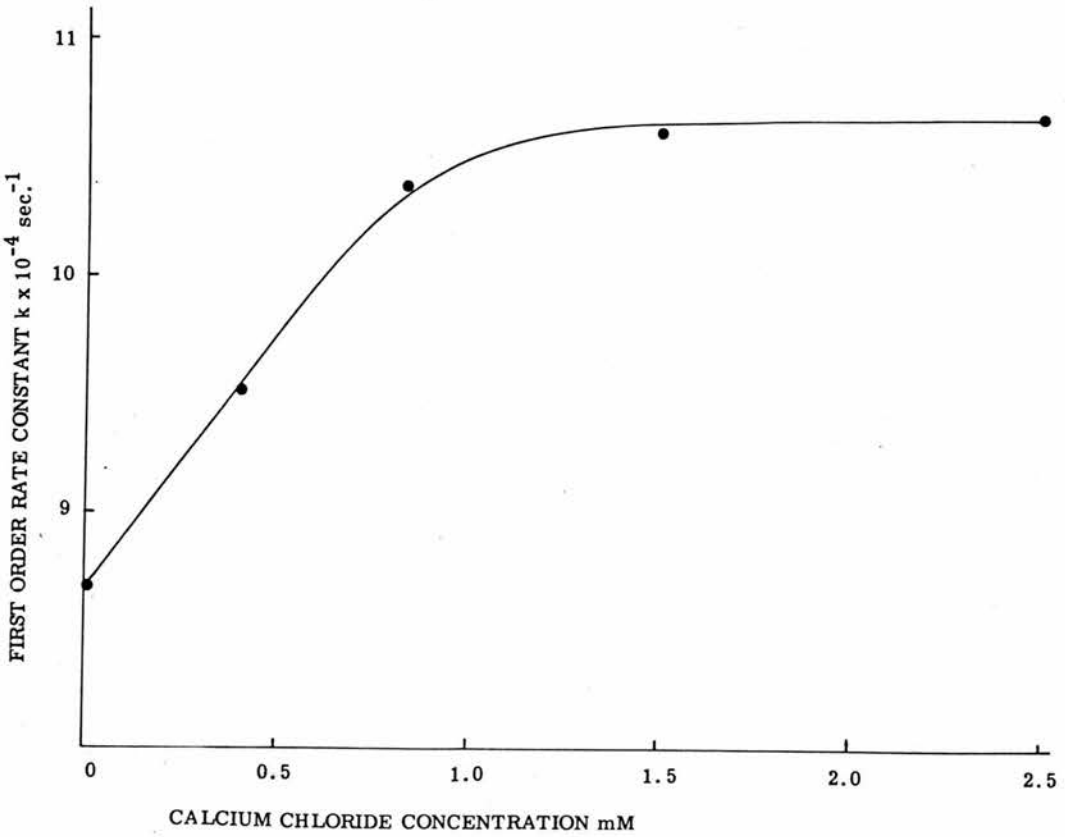


Fig. 4.12. THE EFFECT OF CALCIUM CHLORIDE CONCENTRATION ON THE FIRST ORDER RATE CONSTANT OF CHOLESTEROL INDUCED LOW TO HIGH SPIN STATE TRANSITION OF CYTOCHROME $P450_{scc}$

Cholesterol (final concentration 100 μM), stock solution 2 mM in propylene glycol, was added at time zero to a 1 ml spectrophotometer cuvette containing low spin cytochrome $P450_{scc}$ and the absorbance change (385-420 nm) recorded with time. The cytochrome $P450_{scc}$ concentration was 1.07 nmoles/ml in 20 mM Tris hydrochloride buffer pH 7.0 at the reaction temperature 20°C. Calcium chloride, stock solution 1 M in distilled water, was added 5 mins prior to the addition of cholesterol. The first order rate constants were calculated and plotted against calcium chloride concentration.

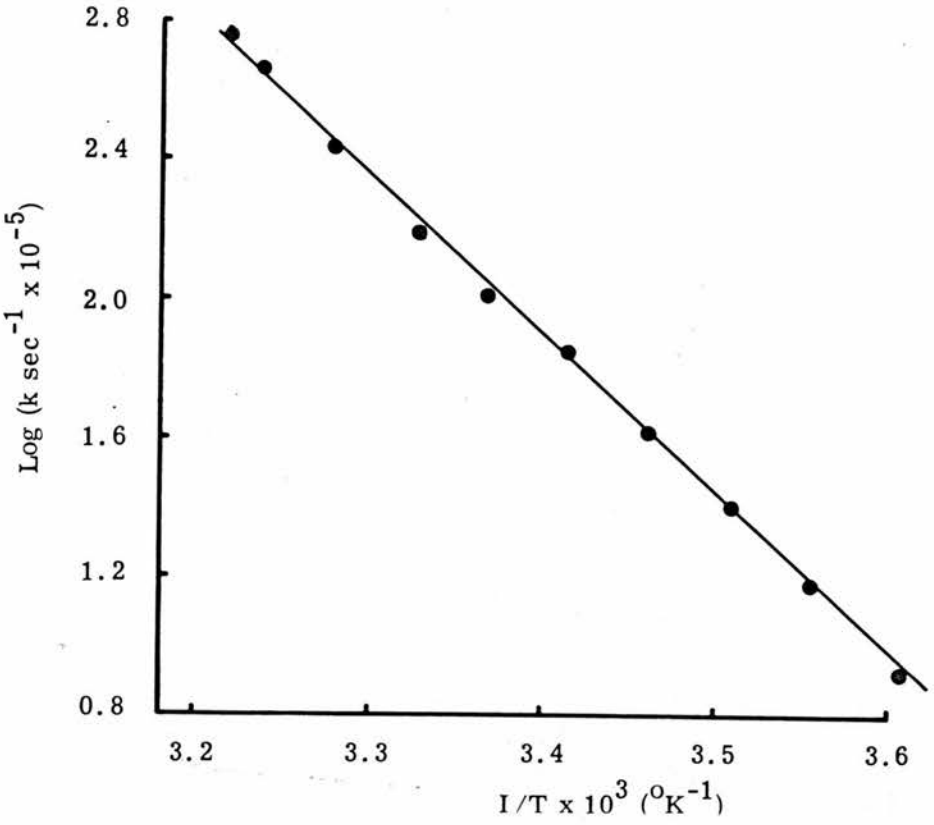


Fig. 4.13. THE RELATIONSHIP OF THE LOGARITHM OF THE FIRST ORDER RATE CONSTANT OF CHOLESTEROL INDUCED LOW TO HIGH SPIN STATE TRANSITION OF CYTOCHROME $P450_{scc}$ TO THE RECIPROCAL OF THE ABSOLUTE TEMPERATURE

The concentration of low spin cytochrome $P450_{scc}$ was 1.7 nmoles/ml in 10 mM potassium phosphate buffer pH 7.4 with 1 mM EDTA and cholesterol was added at time zero. The temperature of reaction T was varied over the range 4-37^{o}C. The absorbance change (385-420 nm) was recorded with time and the first order rate constant k calculated for a particular temperature. The plot of log k against I/T is linear with a gradient of E/2.303R. The activation energy calculated from this plot was 88 kJ mole^{-1}.

mitochondria has been reported as 63 and 76 kJ mole^{-1} (Paul et al. (1976); Jefcoate (1977)).

Monohydroxylated Cholesterol Analogues

Cholesterol analogues with single additional substituents at various positions on the sterol molecule have been compared with cholesterol with regard to their ability to induce a low to high spin state change on substrate depleted cytochrome P450$_{scc}$. The monhydroxylated sterols can be classified according to the position of the hydroxyl substituent; side chain monohydroxylated sterols (26-, 25-, and 24-hydroxycholesterols); nuclear monohydroxylated sterols 7α, 7β and 19-hydroxycholesterols. Monohydroxylated cholesterols were added in saturating equal amounts at time zero to a low spin substrate depleted preparation of cytochrome P450$_{scc}$. The absorbance change (absorbance 385 nm minus absorbance 420 nm) was plotted against time (Fig. 4.14). Maximal absorbance changes were recorded for each cytochrome P450$_{scc}$-sterol complex at equilibrium, 15 hrs at 20°C, and the percentage low to high spin state change calculated using an extinction coefficient 110 mM^{-1} (Peterson (1971), for the Type I spectral change. First order rate constants were calculated for each sterol analogue based on the maximal absorbance change induced by that sterol at equilibrium (Table 4.2). Initial rates of absorbance for each sterol were calculated and expressed as absorbance change (absorbance 385 nm minus absorbance 420 nm) x 10^{-2} min^{-1}.

DISCUSSION

The interpretation of the association of sterols with partially purified cytochrome P450$_{scc}$ and the extrapolation of the data to cytochrome P450$_{scc}$ within the mitochondrial membrane must be made with caution. However the study of partially purified preparations of cytochrome P450$_{scc}$ and the comparison of this preparation with the membrane

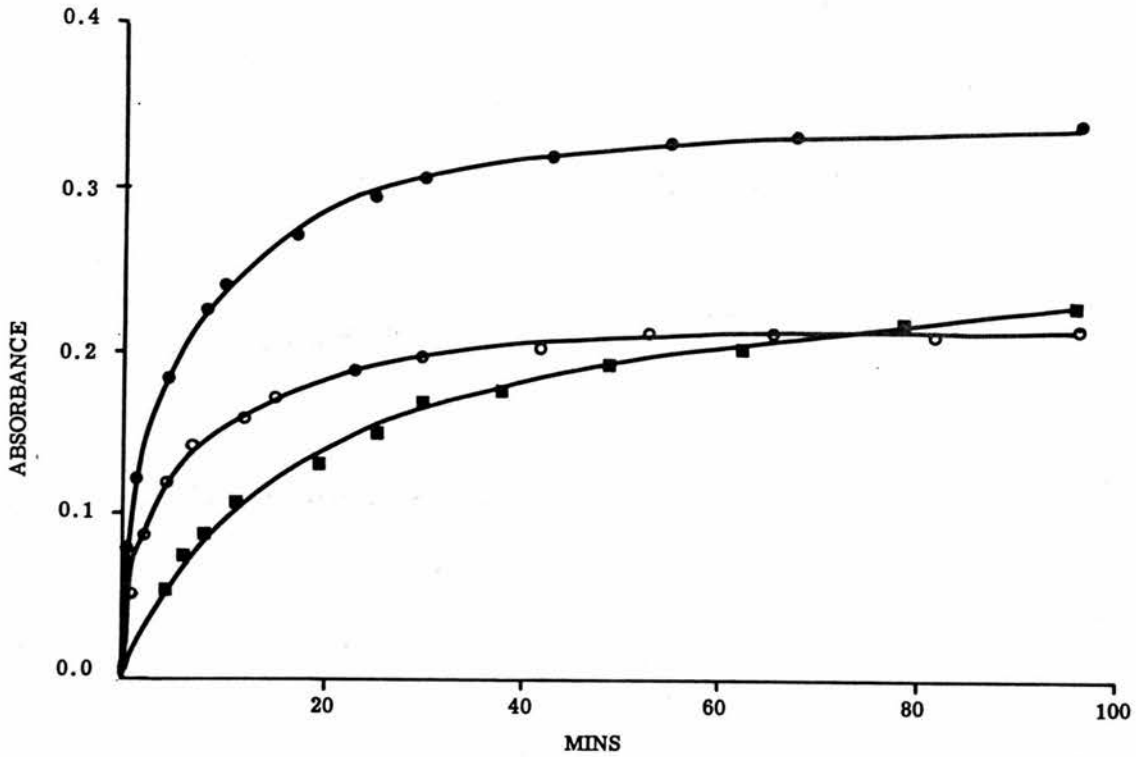


Fig. 4.14. A PLOT OF ABSORBANCE CHANGE (385-420 nm) ON ADDITION OF STEROLS TO LOW SPIN CYTOCHROME $P450_{scc}$ AGAINST TIME

Sterols (final concentration 100 μM), stock solutions 2 mM in propylene glycol, were added at time zero to low spin cytochrome $P450_{scc}$ in a spectrophotometer cuvette. Cytochrome $P450_{scc}$ concentration was 4.8 nmoles/ml in 50 mM potassium phosphate buffer pH 7.4. The reaction temperature was 20°C and the absorbance change was followed with time after the addition of a sterol. The examples shown are cholesterol ■——■ ; a monohydroxylated side chain sterol, 26-hydroxycholesterol O——O and a monohydroxylated nuclear sterol, 7α-hydroxycholesterol ●——●.

TABLE 4.2 Spectral properties of the interaction of monohydroxylated cholesterol analogues with low spin cytochrome $P450_{scc}$

Sterols (100 μM) final concentration) were added at time zero to 1 ml cuvettes containing low spin substrates depleted cytochrome $P450_{scc}$. 4.8 nmoles/ml as determined by the carbon monoxide difference spectrum. Cytochrome $P450_{scc}$ was suspended in 50 mM potassium phosphate buffer pH 7.4, 1 mM EDTA and the reaction temperature was 20°C. The absorbance change (385-420 nm) was plotted against time. Maximal absorbance change for a sterol-cytochrome $P450_{scc}$ interaction was recorded at equilibrium, 15 hrs at 20°C and the percentage low to high spin state change induced by the sterol calculated using an extinction coefficient of 110 $mM^{-1}cm^{-1}$ (Peterson (1971)) for a type 1 change. First order rate constants were calculated using the maximal absorbance change induced by each sterol.

Sterol	First Order Rate Constant R x $10^{-4}sec^{-1}$	Absorbance (385 minus 420 nm) at Equilibrium	Initial Rate Absorbance Change x $10^{-2}min^{-1}$	% Conversion Low to High Spin State at Equilibrium
Cholesterol	3.8	0.38	1.5	70
24-hydroxycholesterol	7.6	0.34	20	63
25-hydroxycholesterol	7.4	0.15	8	27
26-hydroxycholesterol	6.8	0.26	12	51
19-hydroxycholesterol	4.9	0.50	4	82
7α-hydroxycholesterol	9.2	0.42	26	76
7β-hydroxycholesterol	9.0	0.52	32	94

bound cytochrome $P450_{scc}$ may allow an examination of the effects of other membrane components on this haemoprotein. This preparation of partially purified cytochrome $P450_{scc}$ retains features similar to those observed on cytochrome $P450_{scc}$ within the mitochondrial membrane.

The optical changes induced by cholesterol and cholesterol analogues on substrate depleted cytochrome $P450_{scc}$ are similar to those observed on addition of camphor to cytochrome $P450_{CAM}$ (Gunsalus et al. (1972); Peterson (1971)), and are interpreted as a low to high spin state transition of the haemoprotein (Tsai et al. (1970). The association of substrate to ferric cytochrome P450 is considered to be the initial event in the catalytic turnover of the haemoprotein (Estabrook et al. (1972)).

The first order rate constant of cholesterol induced low to high spin state transition of partially purified cytochrome $P450_{scc}$ is $k = 7.5 \times 10^{-4}$ sec^{-1}, a similar value was obtained for cholesterol association to cytochrome $P450_{scc}$ in intact mitochondria (Mason et al. (1978a)). Cholesterol association with cytochrome $P450_{scc}$, in terms of a low to high spin state change is slow both in intact mitochondria and in this partially purified preparation when compared with the first order rate constant of 7000 sec^{-1} for camphor binding to cytochrome $P450_{CAM}$ (Gunsalus et al. (1972)). The importance of the cholesterol side chain cleavage reaction as a rate limiting event in steroidogenesis has been emphasised by Stone and Hechter (1954) and is directly or indirectly under the influence of ACTH which may act indirectly on intramitochondrial movement or binding of cholesterol to cytochrome $P450_{scc}$ (Mason et al. (1978a); Simpson et al. (1978)).

Potassium chloride appears to inhibit the cholesterol induced low to high spin state transition of cytochrome $P450_{scc}$. The results

suggest that at least two inhibitory processes are involved and that the effect with the lower K_i value is antagonised by cholesterol. The effect of potassium chloride on camphor binding to cytochrome $P450_{CAM}$ is to lower the spectral dissociation constant (Peterson (1971)).

Calcium ions were found to increase the rate of association of cholesterol to partially purified cytochrome $P450_{scc}$ as measured by the low to high spin state change of the haemoprotein. Simpson et al. (1974) observed, in rat adrenal mitochondria, an increase in the Type I spectral change with substrates of cholesterol side chain cleavage in the presence of calcium ions. Mason et al. (1978b) propose that calcium ions and certain basic proteins and peptides alter the lipid phase separation of mitochondrial membrane resulting in displacement of cholesterol to a site nearer the cholesterol desmolase enzyme system. A similar mechanism could operate in the calcium ion stimulation of the rate of cholesterol association with partially purified cytochrome $P450_{scc}$ as the preparation has trace phospholipid as contaminant.

Mason et al. (1978a) propose that sterols with non-polar alkyl side chains like cholesterol approach the active site of the sterol desmolase in intact mitochondria in a different manner to sterols with polar side chains. These observations have been extended to the spectral interactions of side chain, nuclear and nuclear angular methyl monohydroxylated cholesterol analogues with partially purified cytochrome $P450_{scc}$. The first order rate constants of association of the side chain monohydroxylated sterols (24-, 25-, and 26-hydroxycholesterol) to cytochrome $P450_{scc}$ are similar but differ from that of cholesterol (Table 4.2), and the nuclear monohydroxylated sterols (7α, 7β and 19 hydroxycholesterols). This may indicate different patterns of association of these groups of sterols with cytochrome $P450_{scc}$.

The maximal absorbance change induced by a sterol on cytochrome $P450_{scc}$ is sensitive to minor variation in the position of the hydroxyl group within the molecule and to optical isomers of the hydroxyl substituent at the same site. In the case of the side chain monohydroxylated sterols, the maximal absorbance change is of the pattern 24- > 26- > 25-hydroxycholesterol. The configuration of the hydroxyl group at C7 show that 7β-hydroxycholesterol induces a maximal absorbance change greater than 7α-hydroxycholesterol.

The initial velocities of sterol induced absorbance changes with cytochrome $P450_{scc}$ are shown in Table 4.2 and the rates derived for the side chain monohydroxylated sterols show an order 24- > 26- > 25-hydroxycholesterol > cholesterol, a pattern which agrees with the rates of enzymatic activity for these sterols (Mason et al. (1978a)). However if this association between spectral changes and enzymatic activity is retained at equilibrium, the pattern of activity predicted from the spectral data would be cholesterol > 24- > 26- > 25-hydroxycholesterol. The further correlation of spectral perturbations induced by sterols on cytochrome $P450_{scc}$ and the enzymatic activity of the cytochrome $P450_{scc}$-sterol complex is in progress.

SUMMARY

The absorption maximal of low spin, cholesterol depleted cytochrome $P450_{scc}$ are 416, 535 and 568 nm; addition of cholesterol results in the formation of a high spin cytochrome $P450_{scc}$-cholesterol complex and a decrease in absorbance at 416 and 568 nm, an increase in absorbance at 392 and 648 nm with isosbestic points at 406, 457, 533 and 588 nm.

The affinity of cholesterol binding to cytochrome $P450_{scc}$ has been determined from the spectral dissociation constant Ks. The spectral dissociation constant is related to the concentration of cytochrome $P450_{scc}$; the equilibrium constant for cholesterol association is K_{equil} =

$0.43 \pm 0.03 \times 10^{6}\ M^{-1}$ in 10 mM potassium phosphate buffer pH 7.4 at 20°C. Potassium chloride appears inhibitory to cholesterol induced low to high spin state change of cytochrome $P450_{scc}$.

The first order rate constant of cholesterol association with cytochrome $P450_{scc}$ is $k = 7.5 \times 10^{-4}\ sec^{-1}$ at 20°C and is decreased by the presence of potassium chloride and increased by calcium chloride. The activation energy for the first order rates of cholesterol association to cytochrome $P450_{scc}$ is 88 kJ $mole^{-1}$.

The first order rate constants of association of side chain monohydroxylated sterols (24-, 25- and 26-hydroxycholesterol) to cytochrome $P450_{scc}$ are similar to one another but differ from that of cholesterol, or the nuclear monohydroxylated sterols, 7α and 7β - hydroxycholesterol and 19-hydroxycholesterol. The maximal absorbance change induced and the initial rate of association however is dependent on minor variations in the position of the hydroxyl group within the sterol and to the optical isomers at the same site.

CHAPTER 5

REDUCTION AND OXYGENATION OF THE CYTOCHROME $P450_{scc}$-CHOLESTEROL COMPLEX

INTRODUCTION

The reduction of oxidised cytochrome $P450_{CAM}$ indicates that one electron is accepted per haem group in this reaction whether the natural electron donor (Gunsalus et al. (1972)) or an artificial donor is used (Peterson (1971)). However determination of the stoicheiometry of reduction of purified rat liver microsomal P450 fraction LM2 suggested that two electrons per haem were accepted (Guengerich et al. (1975)) although potentiometric measurements by the same authors gave a value of n = 1 (van der Hoeven et al. (1974)). Repetition of these experiments using dithionite as reductant to rat liver microsomal cytochrome P450 fractions LM2 and LM4 and cytochrome $P450_{CAM}$ showed that each of these proteins accepted and donated one electron (Peterson et al. (1977)). However the authors point out irreproducible values as high as n = 2 could still be obtained and that a lag phase occurred in the titration curve with dithionite as reductant to purified rat liver microsomal cytochrome P450 before stoicheiometric reduction was obtained. A more recent report (Cooper et al. (1977)) titrating rat liver microsomes with dithionite showed a stoicheiometry of n = 1 and no lag phase in the reduction of cytochrome P450. In view of the above results and the potentially higher electron equivalence for conversion of substrate to product in the side chain cleavage of cholesterol to pregnenolone compared to other cytochrome P450 dependent reactions; a study of the stoicheiometry of reduction of oxidised cytochrome $P450_{scc}$ was undertaken.

Gunsalus et al. (1972) and Estabrook et al. (1972) described a spectral complex of molecular oxygen and ferrous cytochrome $P450_{CAM}$ and in the same year Estabrook et al. (1972) observed a spectral intermediate in liver microsomes during the catalytic cycle which was ascribed to the

oxygenated form of reduced cytochrome P450. The activation of molecular oxygen by cytochrome P450 is crucial to our understanding of the chemical nature of mixed function oxidation and in view of the potentially more complex nature of the cholesterol side chain cleavage reaction, the formation, autoxidation and hydroxylation reactions of oxygenated cytochrome $P450_{scc}$ have been investigated.

RESULTS

Stoicheiometry of Reduction of Cytochrome $P450_{scc}$

Cytochrome $P450_{scc}$-cholesterol complex with catalytic amounts of NADPH adrenodoxin reductase and adrenodoxin (Table 5.1), under an atmosphere of carbon monoxide, was titrated with NADPH. The increase in absorbance at 446 nm was followed as a function of time after each addition of NADPH and the spectra recorded after no further change in absorbance had occurred for a period of 15 min (Fig. 5.1). Under these conditions reduction of cytochrome $P450_{scc}$-cholesterol complex was slow taking 60 min to reach completion. These results plotted as nmoles cytochrome $P450_{scc}$-cholesterol reduced against nmoles NADPH added (Fig. 5.2) show the number of electrons accepted by cytochrome $P450_{scc}$-cholesterol to be 1.02. Repetition with other preparations of cytochrome $P450_{scc}$-cholesterol gives the number of electrons accepted, standard deviation and number of experiments as 1.05 ± 0.04.

After complete reduction, the carbon monoxide complex of cytochrome $P450_{scc}$-cholesterol has absorption maxima of 363, 446 and 550 nm and isosbestic points to the ferric cytochrome $P450_{scc}$-cholesterol form at 350, 425, 534 and 580 nm (Fig. 5.1).

The following observations were made during the reduction of low spin, substrate depleted cytochrome $P450_{scc}$; the extent of reduction was incomplete even at a ten-fold molar excess of NADPH; the rate of reduction was slow and the titration curve showed no definite end point (Fig. 5.3).

TABLE 5.1 Properties of cytochrome $P450_{scc}$ preparation

NADPH adrenodoxin reductase activity was measured by the method of Omura et al. (1966) and expressed as nmoles 2,6-dichlorophenolindophenol reduced/min/nmole cytochrome P450.

Absorption maxima	416, 535 and 568 nm
Absorption maxima (cholesterol complex)	392 and 648 nm
Specific content	6.2 - 8.1 nmoles cytochrome P450/mg protein
Cytochrome P450: cholesterol	1:0.01 molar ratio
Cytochrome P450: adrenodoxin	1:0.01 molar ratio
Cytochrome P450: phospholipid	1:3-6 molar ratio
Cytochrome P450: cholate	1:3 molar ratio
NADPH adrenodoxin reductase	less than 2% mitochondrial activity
Molecular weight	200,000
Enzymatic activity	cholesterol side chain cleavage No 11β hydroxylase activity detected.

TABLE 5.2 Comparison of the principal absorption bands in the spectrum of oxygenated cytochrome $P450_{scc}$-cholesterol complex with other oxygenated haemoproteins

Oxyhaemoglobin	415	541.5	576	Sidwell et al. (1938)
Oxymyoglobin	418	544	582	Yamazaki et al. (1964)
Oxyperoxidase	418	546	581	Yokota and Yamazaki (1965)
Oxygenated-trytophan pyrrolase	418	545	580	Ishimura et al. (1970)
Oxygenated-cytochrome P450-camphor	418	555	580	Gunsalus et al. (1972) Estabrook et al. (1972)
Oxygenated-cytochrome P450-cholesterol side chain cleavage	418	555	-	Hume and Boyd (1978)

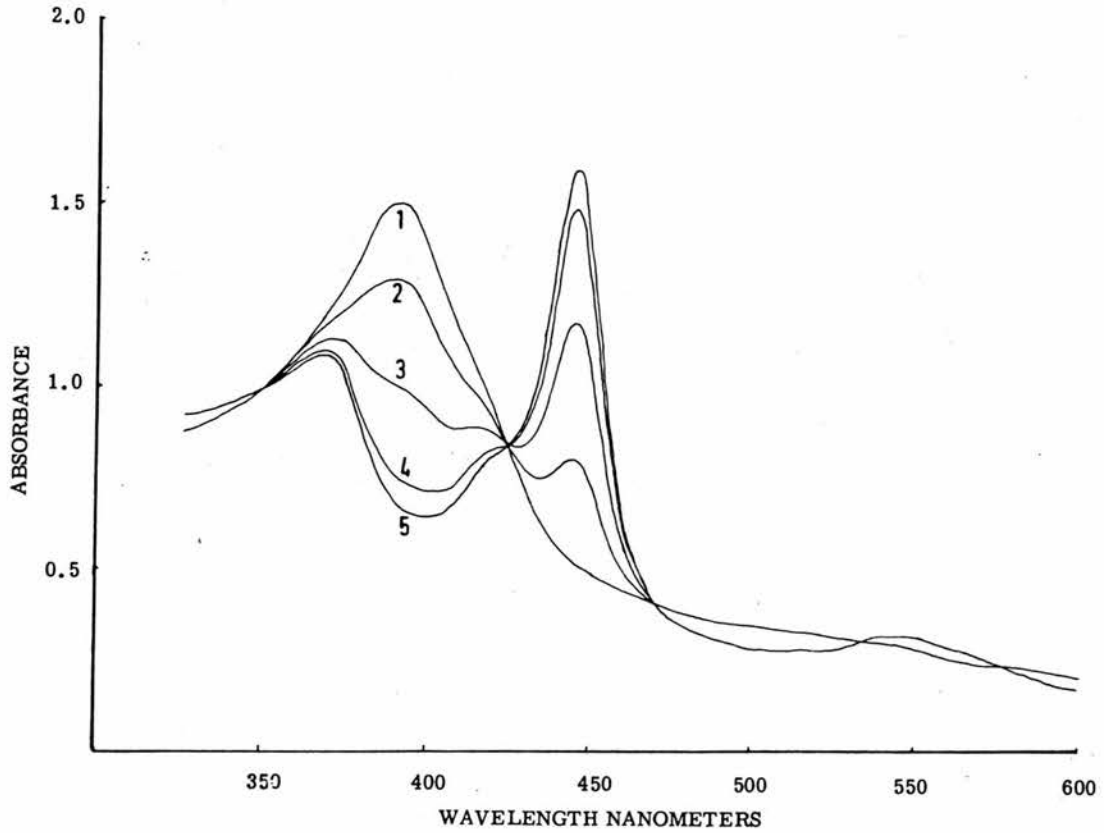


Fig. 5.1. TITRATION OF CYTOCHROME $P450_{scc}$ CHOLESTEROL COMPLEX WITH NADPH UNDER AN ATMOSPHERE OF CARBON MONOXIDE

Cytochrome $P450_{scc}$ at a concentration of 12.25 nmoles/ml in 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA with cholesterol added to 100 nmoles/ml was placed in an anaerobic spectrophotometer cuvette, total volume 2.5 ml, and bubbled with oxygen free nitrogen for 30 min; the cuvette was then bubbled with oxygen free carbon monoxide for 15 min. The specific content of cytochrome $P450_{scc}$ was 6.8 nmoles/mg protein. The sample was titrated with aliquots of an anaerobic solution of NADPH and spectra recorded after each addition when reduction was complete:

curve 1 - no addition of NADPH,

curve 2 - 2.14 nmoles/ml NADPH added,

curve 3 - 4.28 nmoles/ml NADPH added,

curve 4 - 6.62 nmoles/ml NADPH added,

curve 5 - 8.56 nmoles/ml NADPH added.

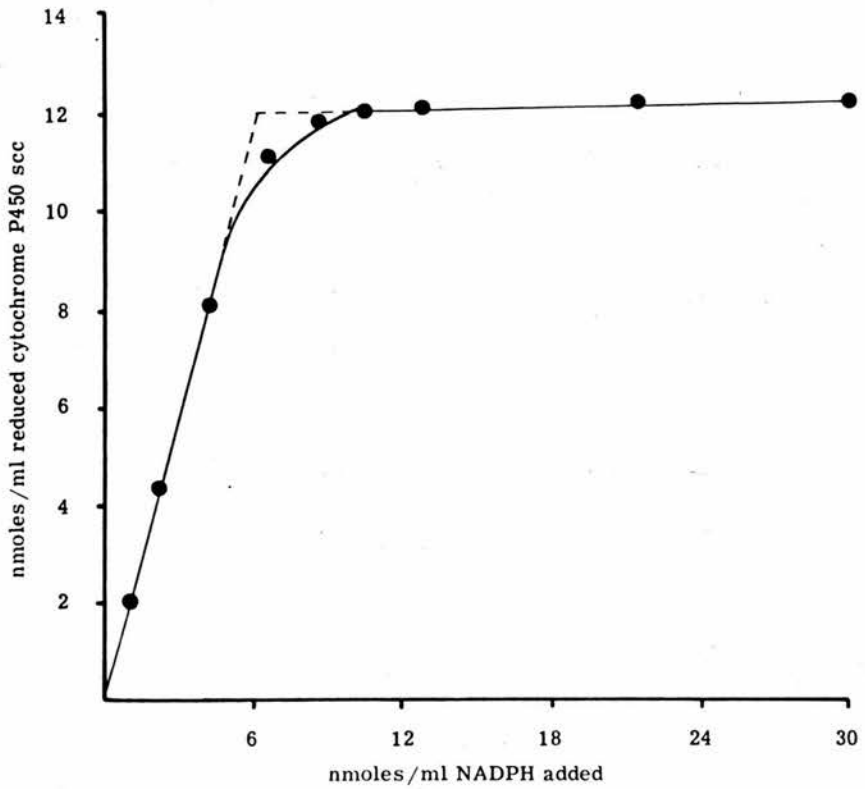


Fig. 5.2. RELATIONSHIP BETWEEN REDUCED CYTOCHROME $P450_{scc}$-CHOLESTEROL COMPLEX AND AMOUNTS OF NADPH ADDED

The data was taken from Fig. 5.1 using the extinction coefficient of 106 $mM^{-1}cm^{-1}$ (Gunsalus et al. (1972)) for the absolute spectrum of the reduced carbon monoxide complex of cytochrome P450 at 446 nm.

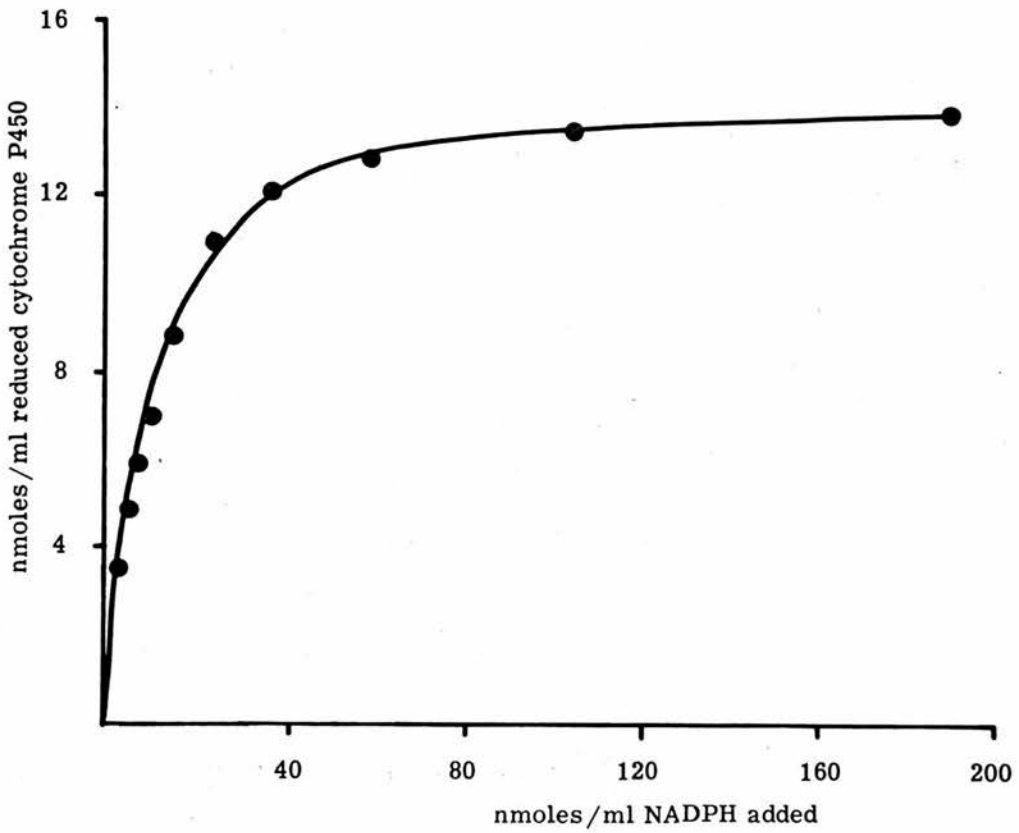


Fig. 5.3. RELATIONSHIP BETWEEN LOW SPIN SUBSTRATE DEPLETED CYTOCHROME $P450_{scc}$ AND AMOUNTS OF NADPH ADDED

The data were obtained by the method described in Fig. 5.2. The cytochrome P450 concentration was 15.1 nmoles/ml and the data emphasises the lack of definite end point of reduction and the molar excesses of NADPH required to effect reduction.

Oxygenated Cytochrome $P450_{scc}$-Cholesterol Complex

Cytochrome $P450_{scc}$-cholesterol was reduced, under anaerobic conditions, with stoicheiometric amounts of sodium dithionite to produce a ferrous form with absorption maxima at 411 and 545 nm. Oxygenation of ferrous cytochrome $P450_{scc}$-cholesterol results in the appearance of a spectral species with absorption maxima at 355, 418 and 555 nm. These spectral characteristics distinguish it from other species of the enzyme with marked similarity to oxygenated cytochrome $P450_{CAM}$ (Gunsalus et al. (1972)) and other oxygenated haemoproteins (Estabrook et al. (1972)) (Table 5.2).

Carbon Monoxide Interchange with Oxygen

The replacement of oxygen in the medium of the oxygenated cytochrome $P450_{scc}$-cholesterol complex by carbon monoxide is useful to differentiate between a possible low spin ferric complex and that of a ferrous-oxygenated species. In the former no development of the characteristic red shift of the Soret band to 446 nm of the carbon monoxide would replace oxygen as a ligand. The data in Fig. 5.4 confirms that carbon monoxide can replace oxygen from the oxygenated complex.

Autoxidation of Oxygenated Cytochrome $P450_{scc}$-Cholesterol

In the absence of adrenodoxin, the oxygenated cytochrome $P450_{scc}$-cholesterol complex spontaneously oxidises to high spin ferric cytochrome $P450_{scc}$ with isosbestic points at 409, 495, 535 and 596 nm (Fig. 5.5). The autoxidation can be followed by the decrease in absorbance at 418 nm (Fig. 5.6) and shows an exponential decay with time. The decay of the oxygenated complex follows first order kinetics in that a plot of the logarithm of absorbance at 418 nm, which corresponds to the fraction remaining of the oxygenated complex, against time is linear (Fig. 5.7). First order rate constants can be calculated from

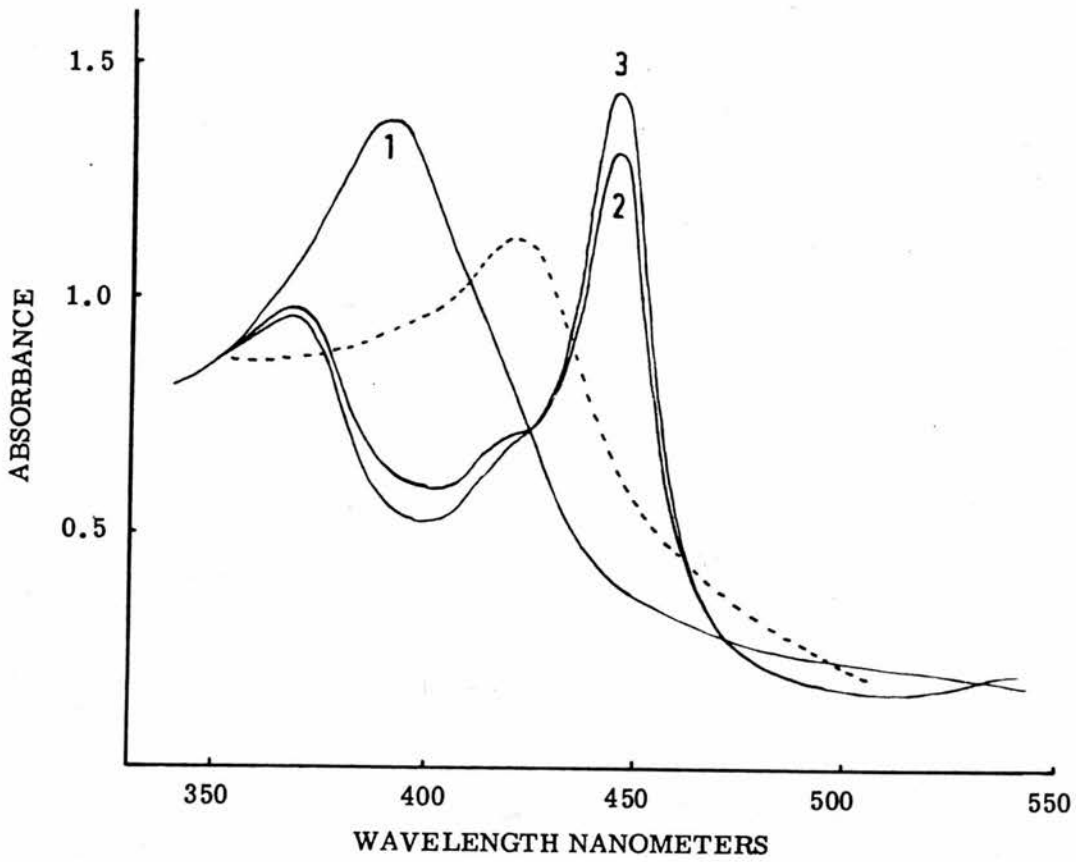


Fig. 5.4. REPLACEMENT OF OXYGEN FROM THE CYTOCHROME $P450_{scc}$-CHOLESTEROL-OXYGEN COMPLEX BY CARBON MONOXIDE.

Cytochrome $P450_{scc}$ concentration was 12.25 μM with 100 μM cholesterol in 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA, curve 1. The solution was reduced with a stoicheiometric amount of dithionite, under anaerobic conditions, and when fully reduced oxygenated with a bubble of oxygen to form the oxygenated complex -------. The solution was then bubbled with 5 ml carbon monoxide and the spectrum recorded, curve 2. Further addition of a grain of sodium dithionite represents full development of the reduced carbon monoxide complex, curve 3.

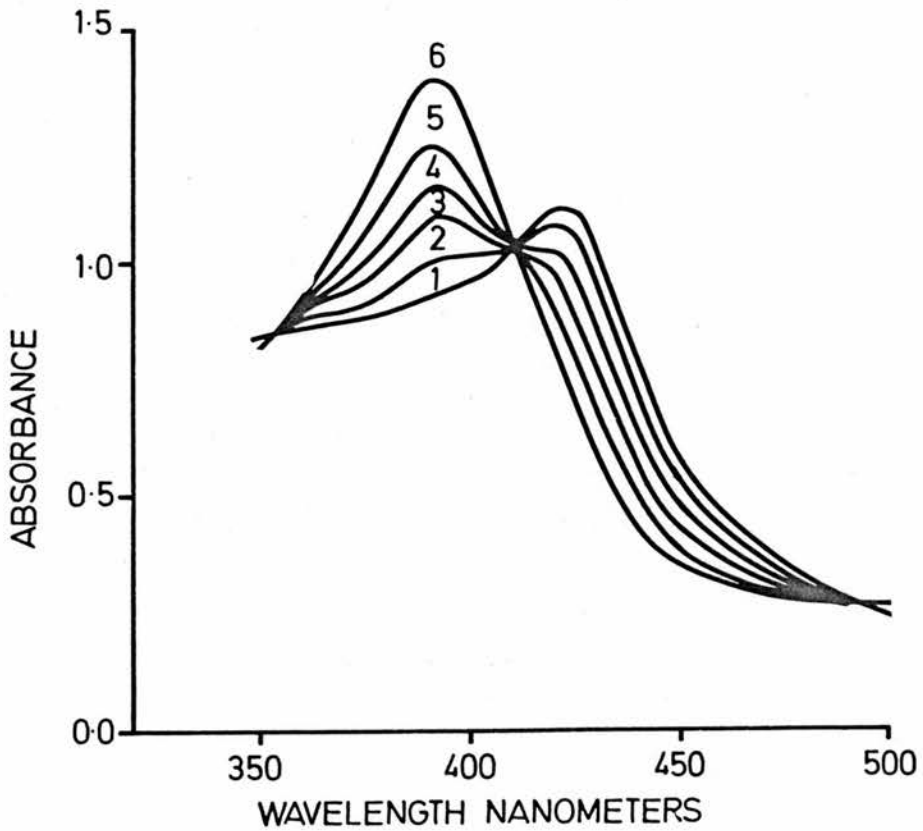


Fig. 5.5. THE AUTOXIDATION OF THE OXYGENATED COMPLEX OF CYTOCHROME $P450_{scc}$-CHOLESTEROL COMPLEX

Cytochrome $P450_{scc}$ at a concentration of 12.5 μM with 100 μM cholesterol in 10 mM potassium phosphate buffer pH 7.4 was reduced stoicheiometrically with sodium dithionite solution. The solution was oxygenated with a bubble of oxygen and the spectrum recorded (1) 5 sec after oxygenation. Repetitive scans were made at (2) 15 sec; (3) 45 sec; (4) 75 sec; (5) 90 sec and (6) 30 min. The spectrum at 30 min was identical to the original ferric enzyme. The temperature of reaction was $4^{o}C$.

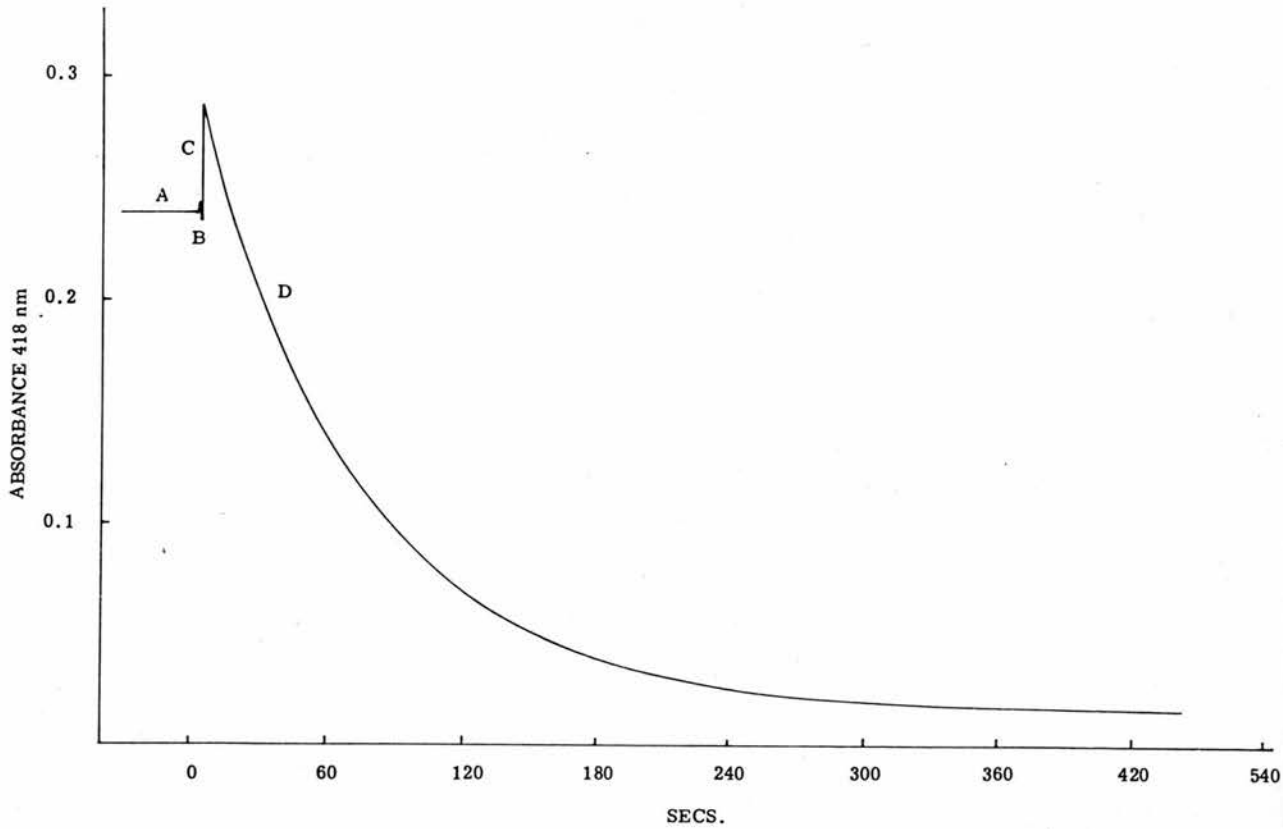


Fig. 5.6. THE AUTOXIDATION OF OXYGENATED CYTOCHROME $P450_{scc}$ WITH TIME AS FOLLOWED BY THE CHANGES IN ABSORBANCE AT 418 nm

A solution of 10 μM cytochrome $P450_{scc}$, with cholesterol added to 100 μM, in 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA was made anaerobic in a spectrophotometer cuvette as described in Methods. The solution was reduced with a stoicheiometric amount of sodium dithionite (stock solution 50 mM in anaerobic 10 mM potassium phosphate buffer pH 7.4) and the extent of reduction was observed by the decrease in absorbance at 390 nm until maximal and constant with time. The absorbance of reduced cytochrome $P450_{scc}$ at 418 nm was then monitored (curve A); the solution was then oxygenated with a bubble of oxygen (point B); the absorbance increased with the formation of the oxygenated complex (curve C) and the subsequent exponential decay to the ferric form observed with time (curve D). The temperature of the reaction was $4^{o}C$.

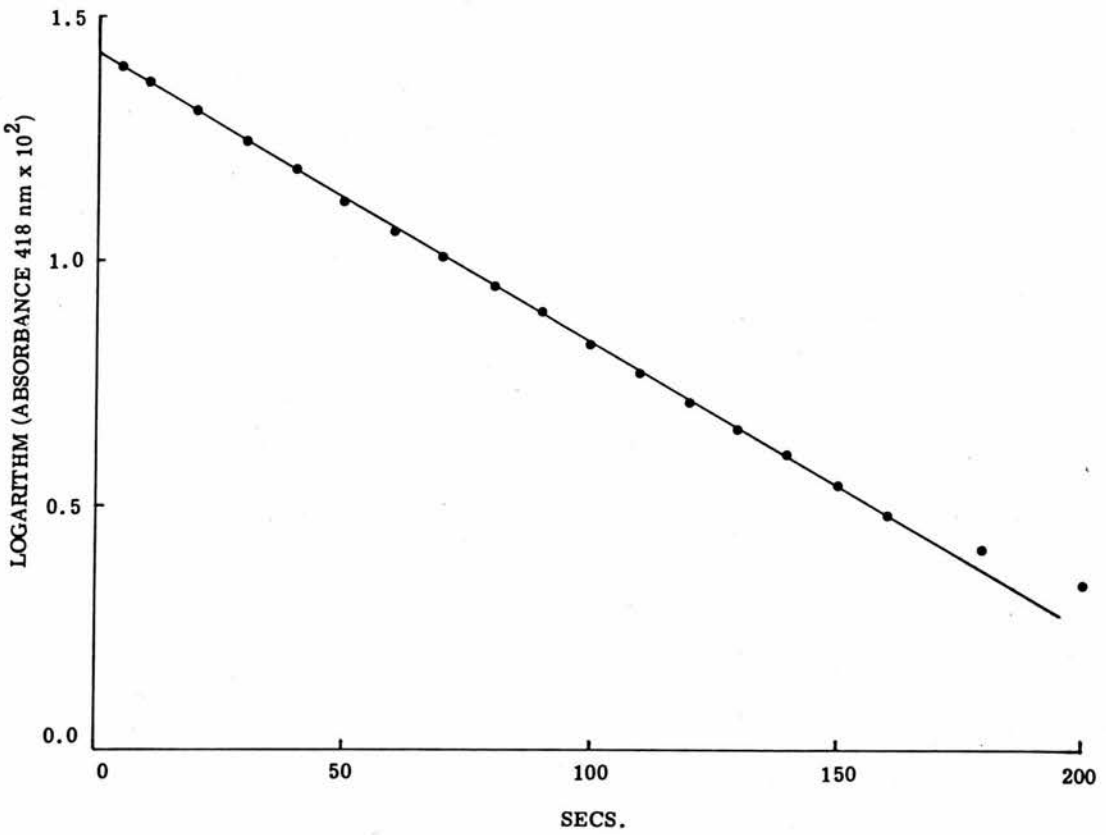


Fig. 5.7. THE AUTOXIDATION OF OXYGENATED CYTOCHROME P450$_{scc}$ AS A PLOT OF LOGARITHM ABSORBANCE AT 418 nm, WHICH CORRESPONDS TO THE FRACTION REMAINING OF OXYGENATED COMPLEX, VERSUS TIME

The data is taken from the decay curve as shown in Fig. 5.6.

this plot using the expression slope = $\frac{-k}{2.303}$. The order of decay is confirmed by the first order rate constants being independent of the initial concentration of oxygenated cytochrome $P450_{scc}$-cholesterol complex and that the initial rate of decay of the complex is proportional to the concentrated of the complex (Fig. 5.8). The rate of decay is constant between preparations with a first order constant, $k = 9.3 \times 10^{-3} \pm 0.26 \times 10^{-3}$ sec^{-1} at $4^{o}C$.

Factors Influencing Autoxidation of Oxygenated Cytochrome $P450_{scc}$

a) pH

The effect of pH, at constant ionic strength and temperature, observed on the first order rate constant of autoxidation of oxygenated cytochrome $P450_{scc}$-cholesterol complex is shown in Fig. 5.9. The influence of pH in the range pH 8.0-7.0 is minimal but below pH 7.0 a marked effect of pH on the first order rate of autoxidation of oxygenated cytochrome $P450_{scc}$-cholesterol exists. The influence of pH below 6.0 was not observed in view of increasing insolubility of cytochrome $P450_{scc}$ as it approaches its isoelectric point. Similar observations of pH dependence of first order rate constants have been made on cytochrome $P450_{CAM}$ (Peterson et al. (1972); Lipscomb et al. (1976)).

b) Temperature

First order rate constants of autoxidation of oxygenated cytochrome $P450_{scc}$-cholesterol were determined over the temperature range $4-25^{o}C$ in 10 mM potassium phosphate buffer pH 7.4 and the logarithm of k plotted against the reciprocal of absolute temperature (Fig. 5.10). The activation energy of autoxidation of cytochrome $P450_{scc}$-cholesterol complex is 75 kJ $mole^{-1}$. The hydroxylated sterols, 25- and 26-hydroxy-cholesterol induce low to high spin state changes in cytochrome $P450_{scc}$ and on anaerobic reduction and subsequent oxygenation form stable oxygenated complexes. The activation energies of autoxidation of the

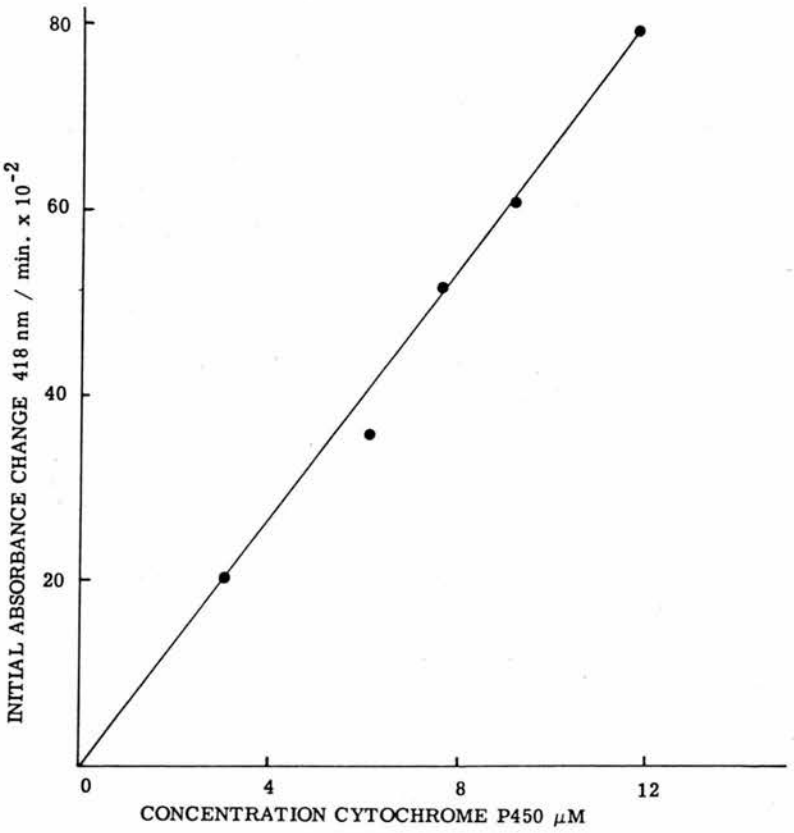


Fig. 5.8. RELATIONSHIP BETWEEN INITIAL VELOCITY OF AUTOXIDATION AND CONCENTRATION OF OXYGENATED CYTOCHROME $P450_{scc}$-CHOLESTEROL COMPLEX

A stock solution of 15 μM cytochrome $P450_{scc}$, with cholesterol (100 μM), was diluted to appropriate concentrations with 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA. The solutions were made anaerobic in a spectrophotometer cuvette as described in Methods and reduced with stoicheiometric amounts of NADPH; stock solution 10 mM in 0.5% sodium bicarbonate. The extent of reduction was observed by the decrease in absorbance at 392 nm and followed until maximal and constant with time. The solutions were oxygenated with a bubble of oxygen and the absorbance at 418 nm followed with time. The initial velocity was calculated from the plot and the initial concentration of oxygenated cytochrome $P450_{scc}$-cholesterol complex calculated from the absorbance 418 nm at time zero using an extinction coefficient of 62 $mM^{-1}cm^{-1}$. The temperature of reaction was $4^{o}C$.

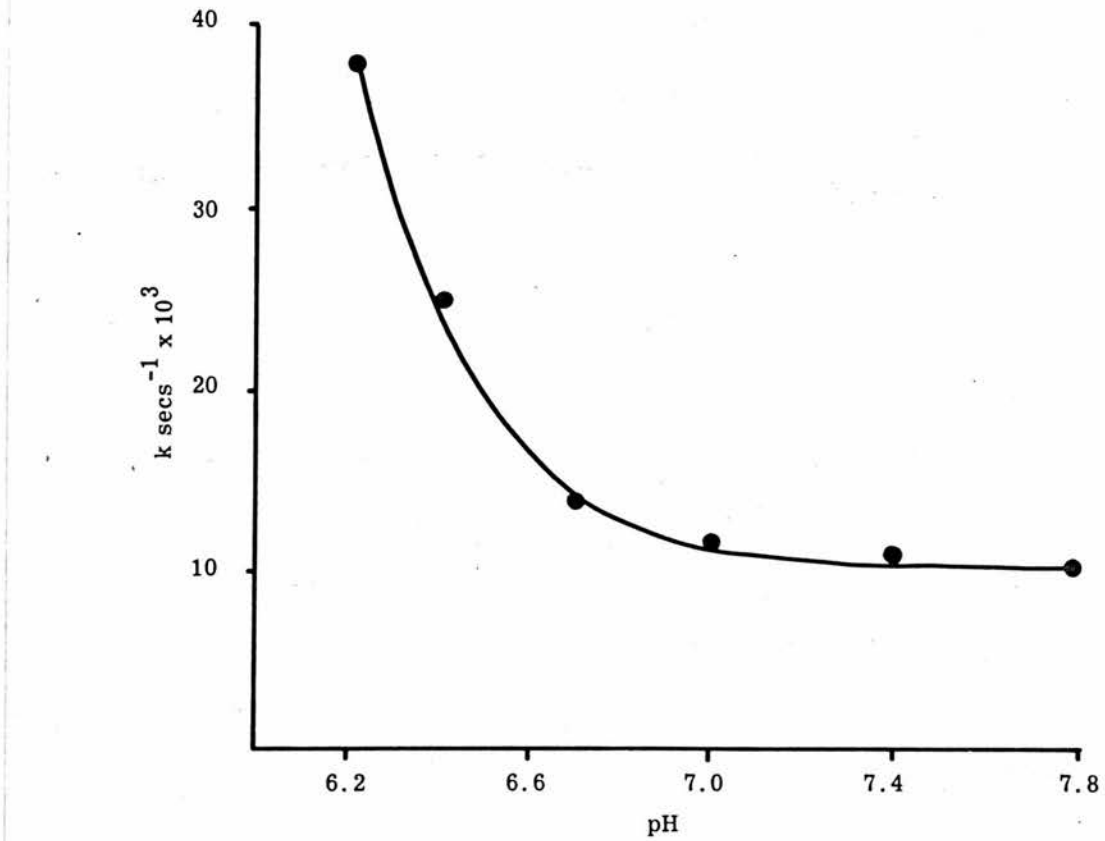


Fig. 5.9. THE EFFECT OF pH, AT CONSTANT IONIC STRENGTH AND TEMPERATURE, ON THE FIRST ORDER RATE CONSTANT OF AUTOXIDATION OF OXYGENATED CYTOCHROME $P450_{scc}$-CHOLESTEROL COMPLEX

First order rate constants were determined as in Figs. 6 and 7 on a 10 μM solution of cytochrome $P450_{scc}$, with cholesterol added to 100 μM, at constant ionic strength 0.1 μ and temperature 4°C but variation in pH between 6.2 and 8.0.

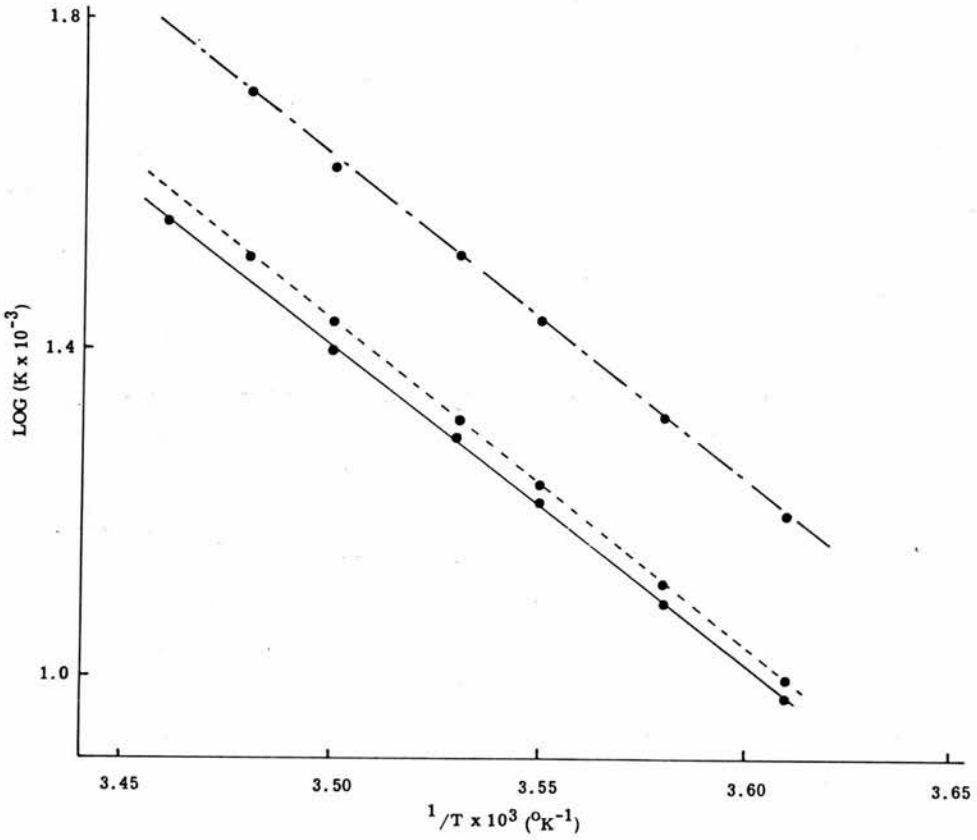


Fig. 5.10. A PLOT OF THE LOGARITHM OF THE FIRST ORDER RATE CONSTANTS OF THE AUTOXIDATION OF THE OXYGENATED CYTOCHROME $P450_{scc}$-STEROL COMPLEX (log k 10^{-3}) VERSUS THE RECIPROCAL OF ABSOLUTE TEMPERATURE (I/T x 10^{3} $^{o}K^{-1}$).

First order rate constants were determined as in Figs. 5.6 and 5.7 on a 10 μM solution of cytochrome $P450_{scc}$, with sterol added to 100 μM, in 10 mM potassium phosphate buffer pH 7.4 with 1 mM EDTA. The temperature was varied over the range 4 to 20°C. The plot of log k against I/T is linear with a gradient of E/2.303R. The activation energy was calculated from this plot. The sterol ligand to cytochrome $P450_{scc}$ was varied: _____ 10 μM cytochrome $P450_{scc}$ with 100 μM cholesterol; ------- 10 μM cytochrome $P450_{scc}$ with 100 μM 25-hydroxycholesterol and - ——— - 10 μM cytochrome $P450_{scc}$ with 100 μM 26-hydroxycholesterol.

oxygenated complexes are not significantly different for 25-hydroxycholesterol and 26-hydroxycholesterol at 76 and 75 kJ mole^{-1} respectively. The first order rate constants for the autoxidation of oxygenated cytochrome P450$_{scc}$-hydroxycholesterol complexes are 10 x 10^{-3}sec^{-1} and 18 x 10^{-3}sec^{-1} at 4^{o}C for 25-hydroxycholesterol and 26-hydroxycholesterol respectively.

c) Ionic strength

Variation of ionic strength and the effect of ionic strength on the first order rate constants of autoxidation was investigated at constant pH and temperature. Potassium chloride was added to cytochrome P450$_{scc}$-cholesterol complex in 10 mM potassium phosphate buffer pH 7.4 and the first order rate constants of autoxidation of the oxygenated complex determined over the range 0.012-0.6 μ. The autoxidation of oxygenated cytochrome P450$_{scc}$-cholesterol shows limited dependence on ionic strength (Fig. 5.11). Similar results can be obtained by adding sodium chloride.

d) Hydrogen peroxide

Titration of ferric cytochrome P450$_{scc}$-cholesterol complex under anaerobic conditions with hydrogen peroxide up to concentrations of 10 mM caused no spectral perturbation of the high spin state. However addition of hydrogen peroxide in stoicheiometric amounts to ferrous cytochrome P450$_{scc}$ caused an immediate oxidation of the ferrous to the ferric state. Addition of hydrogen peroxide accelerates the autoxidation of the oxygenated cytochrome P450$_{scc}$-cholesterol complex. Hydrogen peroxide added simultaneously with oxygen to ferrous cytochrome P450$_{scc}$ allowed calculation of first order rate constants of autoxidation of oxygenated cytochrome P450$_{scc}$-cholesterol complex (Fig. 5.12). In the example shown with a concentration of 10 μM cytochrome P450$_{scc}$-cholesterol; hydrogen peroxide, a concentration of 225 μM, accelerated

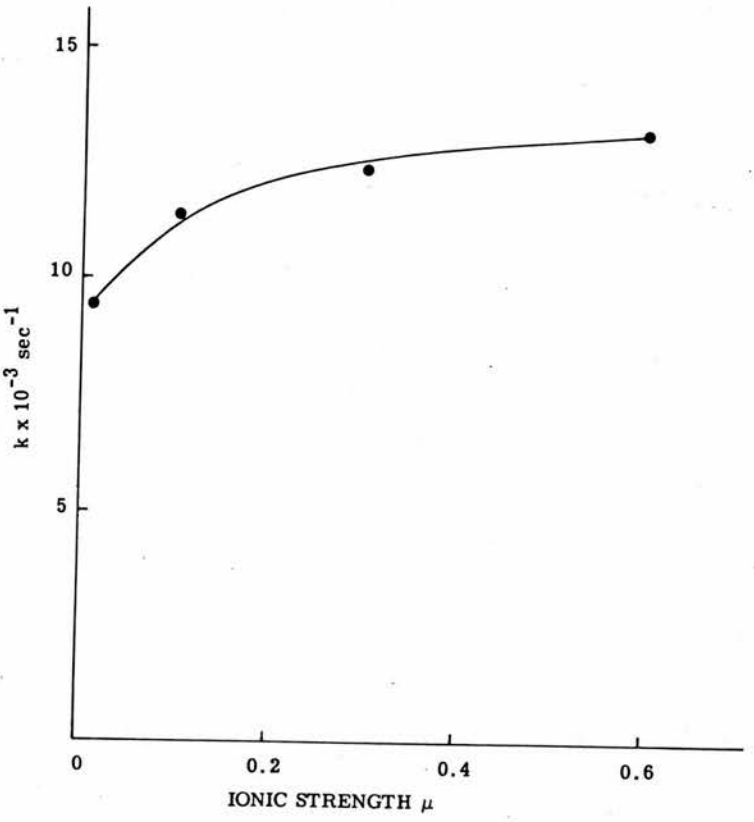


Fig. 5.11. VARIATION OF IONIC STRENGTH AND THE EFFECT ON FIRST ORDER CONSTANTS OF AUTOXIDATION OF OXYGENATED CYTOCHROME $P450_{scc}$

Ionic strength was varied by addition of aliquots of potassium chloride as described in text and the first order constants determined as in Figs. 6 and 7. The concentration of cytochrome $P450_{scc}$ was 10.2 nmoles/ml in 10 mM potassium phosphate buffer with cholesterol added to 100 nmoles/ml and the temperature of reaction was $4^{o}C$.

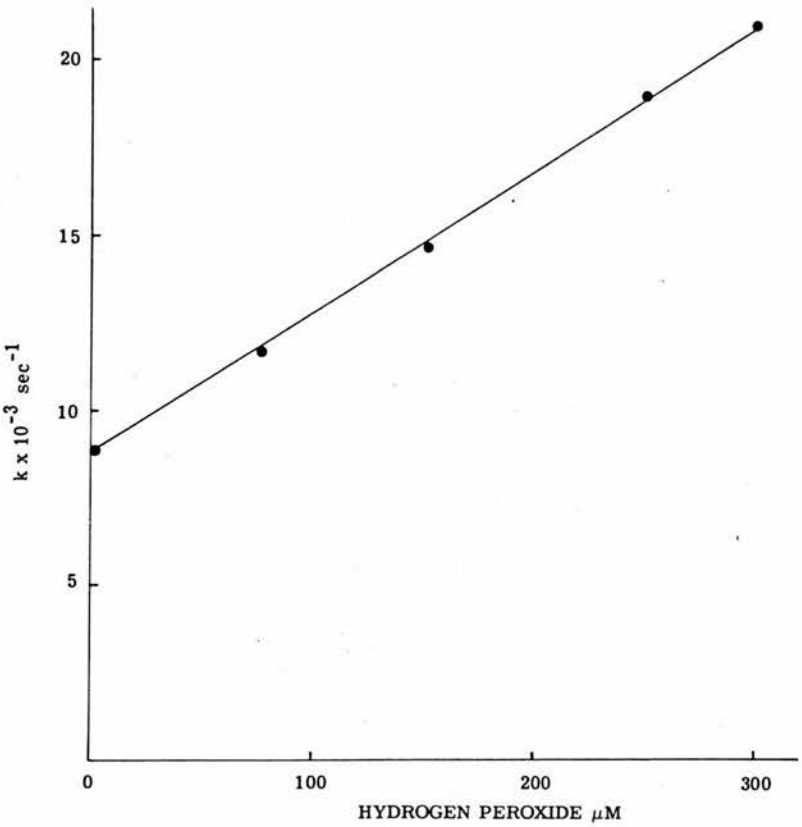


Fig. 5.12. EFFECT OF HYDROGEN PEROXIDE ON THE FIRST ORDER RATE CONSTANTS OF AUTOXIDATION OF OXYGENATED CYTOCHROME $P450_{scc}$-CHOLESTEROL COMPLEX

Cytochrome $P450_{scc}$ at 10.1 μM, with cholesterol added to 100 μM, in 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA and under an atmosphere of nitrogen was reduced stoicheiometrically with NADPH. The cytochrome was oxygenated with a bubble of oxygen and a solution of hydrogen peroxide was simultaneously added; stock solution of hydrogen peroxide 7.6 mM in oxygen free 10 mM potassium phosphate buffer pH 7.4. First order rate constants of autoxidation were determined as in Figs. 5.6 and 5.7 and plotted against concentration of hydrogen peroxide added. The temperature of reaction was $4^{o}C$.

the decay by a factor of two. The low reactivity of hydrogen peroxide with the oxygenated cytochrome $P450_{scc}$-cholesterol complex suggests a small rate constant for cytochrome P450 deoxygenation for otherwise hydrogen peroxide which reacts rapidly with reduced cytochrome P450 would accelerate markedly the conversion to the oxidised form.

e) Catalase

Inclusion of catalase during the reduction/oxygenation cycle of cytochrome $P450_{scc}$-cholesterol complex prolongs the stability of the oxygenated form. Relatively high catalase activity is required to observe this effect and the maximal decrease in the autoxidation rate plateaus at around 30% (Fig. 5.13). These results suggest that hydrogen peroxide is produced during the autoxidation of oxygenated cytochrome $P450_{scc}$ and the peroxide oxidises in part the reduced cytochrome $P450_{scc}$.

Detection of Hydrogen Peroxide During the Autoxidation of the Oxygenated Complex of Cytochrome $P450_{scc}$-Cholesterol Complex

The method of Loschen et al. (1971) was used to continuously monitor hydrogen peroxide production during the autoxidation of oxygenated cytochrome $P450_{scc}$-cholesterol complex. Simultaneous parallel experiments observing the absorbance decrease of the 418 nm band of the oxygenated complex and the fluorescence emission of scopoletin were made. No free hydrogen peroxide was detected.

Similarly the sampling method of Hildebrandt and Roots (1975) taking aliquots of cytochrome $P450_{scc}$ solution during the decay of the oxygenated complex failed to detect free hydrogen peroxide.

Although no spectral complex was observed between hydrogen peroxide and oxidised cytochrome $P450_{scc}$-cholesterol complex, the measurements of catalase like activity in the preparation by the method of Aebi (1974) showed an activity of 200-300 nmoles hydrogen peroxide consumed/nmole cytochrome P450/min. This activity could explain the lack of free

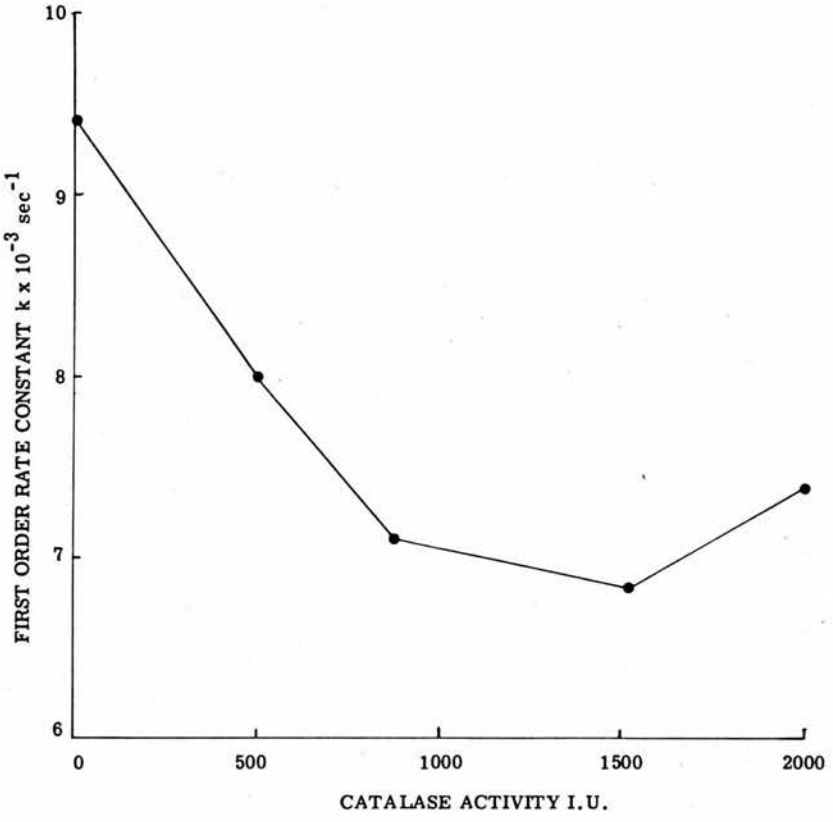


Fig. 5.13. THE EFFECT OF CATALASE ON THE FIRST ORDER RATE CONSTANT OF AUTOXIDATION OF OXYGENATED CYTOCHROME P450$_{scc}$-CHOLESTEROL COMPLEX

Cytochrome P450 concentration was 10.0 μM, with added cholesterol 100 μM, in 10 mM potassium phosphate buffer pH 7.4. A 2.5 ml solution was reduced anaerobically, oxygenated and the first order rate constant of autoxidation determined as previously described. The reaction temperature was 4^{o}C. Catalase activity per incubation is expressed in international units.

hydrogen peroxide during the decay of the oxygenated complex.

Detection of Superoxide Anion During the Autoxidation of the Oxygenated Complex of Cytochrome $P450_{scc}$

The detection of superoxide anion as a decay product of the oxygenated cytochrome $P450_{scc}$-cholesterol complex was investigated. Indirect methods were used, based on changes in absorption spectra of radical scavenging chromophores and the inhibition of these changes by superoxide dismutase. The ideal scavenger should not react chemically with cytochrome P450, it should have a large extinction difference on reaction and should have a maximal absorption change at an isosbestic point of oxidised and oxygenated cytochrome P450.

Cytochrome c, the most commonly used agent (McCord and Fridowich (1969)) is reduced by ferrous cytochrome P450 (Tyson et al. (1972)). Adrenalin is converted to adrenochrome by superoxide anion (Misra and Fridowich (1972)) and has a maximal absorption at 480 nm but a low extinction coefficient at the isosbestic point of cytochrome P450 at 494 nm. Tetranitromethane is reduced to the nitroform anion by the superoxide radical (Beauchamp and Fridowich (1971) and has a maximal absorption at 350 nm. However tetranitromethane reacts with cytochrome P450 to form an amide complex. Similar reactions were observed with nitroblue tetrazolium used by Fridowich (1974) as an indicator of superoxide anion production.

Determination of Cholesterol Oxidation Products During the Autoxidation of Oxygenated Cytochrome $P450_{scc}$

The experimental evidence for the absence of formation of oxidative products of cholesterol during the autoxidation of oxygenated cytochrome $P450_{scc}$ will be presented in Chapter 6. It is sufficient at this stage to discount cholesterol as a possible electron acceptor from the oxygenated complex of cytochrome $P450_{scc}$.

DISCUSSION

In the reduction of purified cytochrome $P450_{CAM}$ one electron is accepted per haem (Gunsalus et al. (1972); Peterson (1971)). The stoicheiometry of reduction of purified rat liver microsomal cytochrome P450 suggested two electrons accepted per haem (van der Hoeven et al. (1974)) repetition of the titrations (Peterson et al. (1977)) and results from liver microsomal studies (Cooper et al. (1977)) give a value of one. The evidence presented suggests that cytochrome $P450_{scc}$-cholesterol is a one electron acceptor. The observations made on reduction of low spin, substrate depleted cytochrome $P450_{scc}$ may indicate a change in redox potential on binding cholesterol similar to that described for cytochrome $P450_{CAM}$ (Gunsalus et al. (1973)).

The absorption spectrum of cytochrome $P450_{scc}$ shows a similarity to that described for the oxygenated complex of cytochrome $P450_{CAM}$ (Gunsalus et al. (1972)) and to other oxygenated haemoproteins (Estabrook et al. (1972)). The oxygenated complexes of cytochrome $P450_{CAM}$ and cytochrome $P450_{scc}$ show similar patterns of stability to pH changes (Peterson et al. (1972); Lipscomb et al. (1976)) variations in ionic strength (Peterson et al. (1972)), changes in temperature (Lipscomb et al. (1976)) and minor modifications in substrate structure (Lipscomb et al. (1976)). However oxygenated cytochrome $P450_{scc}$ is more rapidly autoxidised than cytochrome $P450_{CAM}$ (Peterson et al. (1972); Lipscomb et al. (1976)). Interspecies variation of structure, as in the case of oxyhaemoglobins with differing rates of autoxidation, may be an intrinsic cause of this difference in rate. However the purity, as expressed in nmoles cytochrome P450/mg protein, is less in the cytochrome $P450_{scc}$ preparation compared to that of cytochrome $P450_{CAM}$ as described by Lipscomb et al. (1976) and Peterson et al. (1972). This leaves the possibility of redox acceptors or modifiers unknown influencing the

autoxidation rate as the non-haem iron contents of these different cytochrome preparations are comparable. The rate of autoxidation of oxygenated cytochrome $P450_{CAM}$-camphor complex quoted by Lipscomb et al. (1976) is $2.5 \times 10^{-3} sec^{-1}$ and that by Peterson et al. $3.4 \times 10^{-4} sec^{-1}$ at $4^{o}C$. The reasons for this wide variation in rate for a similar enzyme are unkown at this time.

The spectral correlations of the oxygenated form of reduced cytochrome P450 from liver microsomes to other oxygenated proteins in particular cytochrome $P450_{CAM}$ and $P450_{scc}$ is less clear. Estabrook et al. (1972) described a spectral intermediate in the catalytic cycle of cytochrome P450 which required oxygen, NADPH, and a Type I hydroxylatable substrate with absorption maxima in the difference spectrum of 440 and 590 nm, correcting for cytochrome b_5 absorption. Further evidence for the occurrence of an oxyferrous complex in liver microsomes came from Rosen et al. (1973) who showed by flash photolysis experiments that oxygen and carbon monoxide are competitively bound to the cytochrome. Guengenich et al. (1976) described, using purified liver microsomal cytochrome P450, that during the steady state of enzymatic activity a spectral complex was formed with difference maxima at 442, 560 and 588 nm similar to that described by Estabrook et al. (1972). This spectral species was named Complex II by the authors, was relatively stable with a life of 5 mins before it decayed. The authors again described a requirement for a Type I substrate whether benzphetamine, cyclohexane, nitroanisole or hexobarbital; an NADPH source and molecular oxygen. During rapid reaction studies with the same system the authors noted another spectral complex with maxima in the difference spectra of 430 and 450 nm, Complex I in the terminology of Estabrook et al. (1972) which decayed rapidly in 10 msec to give Complex II. The Soret

maxima of the absolute spectra of purified liver microsomal cytochrome P450 are useful for comparison to those of cytochrome $P450_{CAM}$ and $P450_{scc}$: P450 LM oxidised 418 nm; P450 LM reduced 414; P450 LM Complex I 423 nm and P450 LM Complex II 421 nm.

After complete reduction of cytochrome P450 by a slight excess of NADPH under an atmosphere of carbon monoxide the spectrum shows similarities to carbonmonoxy complexes of cytochrome P450 from other sources, with absorption maxima at 368, 446, and 550 nm and isosbestic points to the ferric high form at 350, 425, 471, 534 and 580 nm. The ratio of absorbance of the 446 nm Soret band to that of the 550 nm band for the carbon monoxide complex of the reduced form is 8.4 which corresponds well with a ratio of 8.2 for $P450_{CAM}$ (Gunsalus et al. (1972); 8.3 for the LM2 fraction of purified rat liver microsomal P450 (Peterson et al. (1977) and values between 8 and 9 from previously published spectra of bovine adrenal mitochondrial P450 (Takemori et al. (1975). Hanson and co-workers (1976) have shown that the optical transition in the near U.V. region of the carbon monoxy $P450_{CAM}$ spectrum at 363 nm has the same integrated intensity as the Soret B (π-π^*) band at 446nm. Using single crystal spectroscopy they have shown that this U.V. band has the same polarisation as the Soret band showing that this U.V. band has the same symmetry. The total integrated intensity of the carbon monoxide $P450_{CAM}$ bands at 446 and 363 nm is within 10% of the total integrated intensity observed within the same region for carbonmonoxyhaemoglobin. However the near U.V. spectrum of this substance has only 40% of the integrated absorption strength of the Soret band and is a N (π-π) band. The authors conclude that these spectral observations are consistent with $P450_{CAM}$ being classified as having a hyperporphyrin structure according to the classification of Buchler (1975). A

common spectral pattern exhibited by a number of hyperporphyrins is two Soret bands one in the near U.V. 350-380 nm region and a second in the 440-480 nm region; a metal to porphyrin charge transfer transition is responsible for the two Soret bands. The pattern of two Soret bands is readily observable in cytochrome $P450_{scc}$ carbon monoxide complex (Fig. 5.1) however the near U.V. band is at slightly longer wavelength, 368 nm, that that reported for cytochrome $P450_{CAM}$, 363 nm (Hanson et al. (1976).

During steady state observation of catalytic turnover of cytochrome $P450_{CAM}$ the dominant spectral species was the oxygenated complex (Gunsalus et al. (1972). First order rate constants of individual reactions of the same enzyme cycle shows that the second electron reduction is the slowest; this reaction may control the balance between autoxidation and hydroxylation pathways of the oxygenated complex. The nature of the products of autoxidation of the oxygenated complex is therefore of interest from consideration of the structure of the oxygenated complex.

Detailed information on the structure of oxygenated cytochrome P450 is lacking and one must reason on the basis of information known from other oxygenated cytochromes. The nature of the ligands to the iron centre of cytochrome P450 have similarities to those of oxyhaemoglobin and oxymyoglobin except that the distal histidine is replaced by a mercaptide anion in cytochrome P450 (Chevion et al. (1977).

On the basis of O-O infra-red stretching frequency, oxygen-metal ion adducts can be subdivided into two classes; class I with O-O frequency of 1100 cm^{-1} called superoxides and class II adducts with O-O frequency of 840 cm^{-1} and called peroxides (Valentine (1973). When the stoicheiometry of the adducts is 1:1 the geometry of the bond must be bent-end on, Fe-O-O or cyclic, Fe<O-O. Oxyhaemoglobin infra-red

stretching frequencies suggest the former structure (Caughey et al. (1975).

In the light of the initial observation that the optical spectra of oxyhaemoglobin and oxymyoglobin bear strong resemblance to those of their respective hydroxy complexes it was suggested that the best electronic description for oxygenated-haem complex is that of low spin ferric iron bound to superoxide anion (Weiss (1964)). This has been supported by X-ray fluorescence (Koster (1972)), Raman spectroscopy and infra-red spectra of oxyhaemoglobin (Caughey et al. (1975). This evidence does not provide a distinction between an ionic $Fe^{3}O_{2}^{-}$, produced by an electron transfer from iron to dioxygen and a covalent Fe-O-O produced by interactions between iron and oxygen. The degree of electron density transferred from iron to dioxygen significantly determines the properties of the complex. If the proposed electronic structure of the oxy-haem complex is that of ferric haem bound to superoxide anion, then the superoxide anion should be able to dissociate as has been demonstrated for shark and mammalian oxyhaemoglobin (Misra and Fridowich (1972); Wever et al. (1973)) as well as cytochrome $P450_{CAM}$ (Sligar et al. (1974)). This does not mean that the superoxide is bound ionically in the original oxygenated haem complex or that the haem iron is tri-positively charged. The structure consisting of superoxide anion bound to low spin ferric haem is only a 'best fit' and clearly other resonance forms must exist.

Consider the equation $Fe^{2+} O\text{-}O \rightleftharpoons Fe^{3+} O\text{-}O^{-}$ as implying charge transfer from iron to oxygen, then a mechanism is suggested by which transfer of more electron density oxygen affinity should be increased and with less transfer of electron density oxygen affinity should be decreased. By substitution of unnatural iron porphyrins these principles have been shown for myoglobin and haemoglobin (Rossi-Fanelli and Antonini (1957);

(Sogita and Yoneyama (1971)). Peisach (1975) has suggested that the ring nitrogen of the proximal imidazole that is not liganded to iron in haemoglobin is in a hydrophobic environment surrounded by three leucines. The hydrophobicity of the environment governs the state of protonation of the imidazole and affects the electron density at the haem iron. The hydrogen bonded will have low affinity for oxygen and vice versa for the non-hydrogen bonded. This suggestion could explain the Bohr effect and the role of allosteric modifiers to indirectly alter hydrophobicity of the proximal imidazole environment.

Haemoglobin involvement as a catalyst of hydroxylation of aniline in the presence of reducing equivalents and oxygen has been well established (Juchau and Symms (1972); Symms and Juchau (1974); (Mieyal et al. (1976)). It has been suggested that aniline increases the hydrophobic character of the active site causing a shift in electron transfer in favour of oxygen and consequently altering the reactivity (Mieyal and Blumer (1976)). Similar speculations could be made for cytochrome P450 where substrate binding, usually highly aromatic or aliphatic in character, stabilise the oxygenated complex (Lipscomb et al. (1976)).

The possible routes of autoxidation of oxygenated cytochrome $P450_{scc}$-cholesterol complex to the ferric state are shown in Fig. 5.14. The dissociation of molecular oxygen leaving the haem in a ferrous state requires the subsequent donation of an electron to either an acceptor group in the protein moiety of the cytochrome, as has been postulated in methaemoglobin formation, or to an acceptor external to the cytochrome. The possibility of formation of the ferric state from the oxygenated cytochrome complex by donation of both electron equivalence and oxygen to the substrate cholesterol must also be considered.

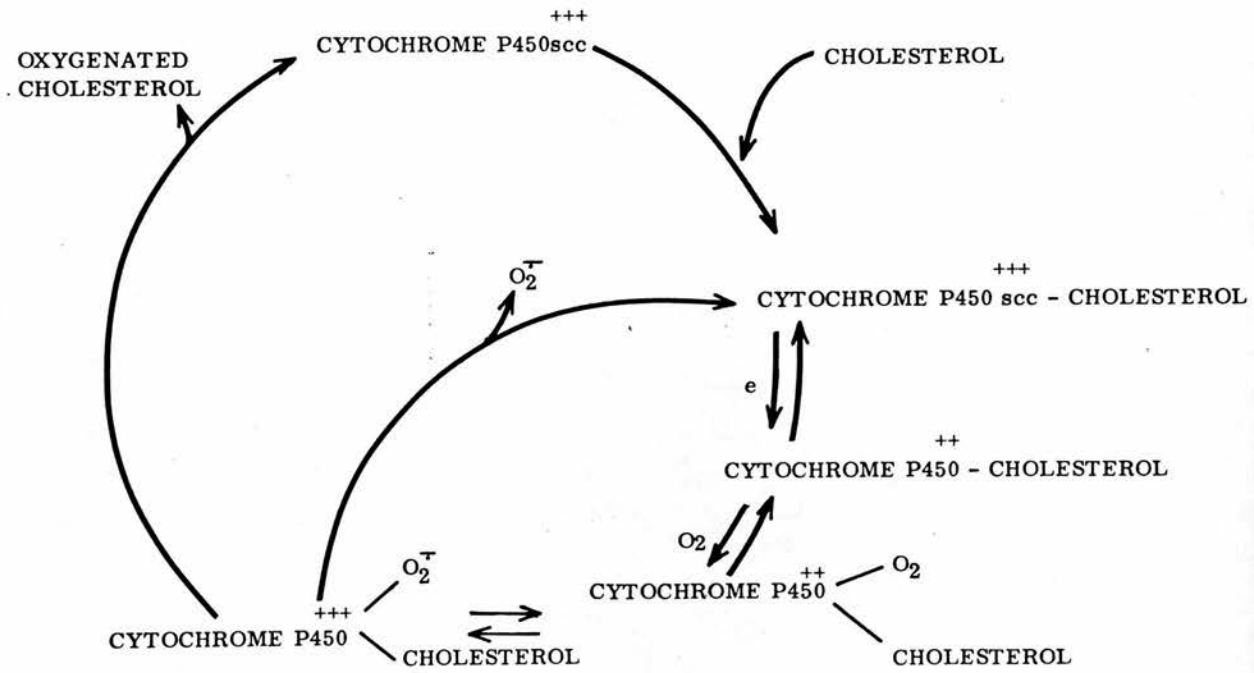


Fig. 5.14. THE POSSIBLE ROUTES OF AUTOXIDATION OF OXYGENATED CYTOCHROME $P450_{scc}$-CHOLESTEROL COMPLEX TO THE FERRIC STATE

However this latter route can be discounted by the failure to detect oxidation products of cholesterol following the autoxidation of the oxygenated cytochrome $P450_{scc}$-cholesterol complex.

If superoxide anion is a product of the autoxidation of oxygenated cytochrome P450 then the subsequent formation of other reactive oxygen species must be considered. It has been suggested that superoxide anion $O_2^{\cdot -}$ spontaneously dismutates (Kahn (1970); Mayeda and Bard (1974)) to singlet oxygen $^1O_2$ as in reaction 1 (Fig. 5.15). The enzyme superoxide dismutase (McCord and Fridowich (1969) catalyzes the dismutation of $O_2^{\cdot -}$ leading to the formation of ground state oxygen (Kahn (1970) rather than $^1O_2$, reaction (2) (Fig. 5.15). In either case, the spontaneous or enzyme catalysed dismutation of $O_2^{\cdot -}$ leads to hydrogen peroxide H_2O_2. It has been postulated that molecular oxygen $O_2^{\cdot -}$ may react with H_2O_2 to produce hydroxyl radicals OH• via Haber-Weiss reaction (Haber and Weiss (1934)), reaction (3) (Fig. 5.15). There is, however, controversy concerning the possibility of this reaction under physiological conditions (Fee and Bergamini (1975); Halliwell (1976)). Direct evidence for OH-generated from reaction 3 has not been demonstrated. Frequently its postulation is based on the inhibiting effect of both superoxide dismutase and catalase on a specific reaction. It has been suggested that under certain conditions, $O_2^{\cdot -}$ may react with H_2O_2 to form O_2 and OH. (Kellogg and Fridowich (1975)). Arneson (1970) has proposed that $^1O_2$ may be generated from a reaction between $O_2^{\cdot -}$ and the hydroxyl radical, as in reaction (4) (Fig. 5.15). This reaction has not been demonstrated under biological conditions. Superoxide radicals are known to dismutase to form peroxide and oxygen in an excited singlet state. Chemiluminescence from singlet oxygen has been observed, the major pathway for de-excitation being a dimeric relaxation of two excited states producing photons with a wavelength in the region of

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow {}^1O_2 + H_2O_2 \quad (1)$$

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow {}^1O_2 + H_2O_2 \quad (2)$$

$$O_2^{\cdot -} + H_2O_2 \rightarrow OH^- + OH. + O_2 \quad (3)$$

$$O_2^{\cdot -} + OH. \rightarrow {}^1O_2 + OH^- \quad (4)$$

Fig. 5.15. THE POSSIBLE REACTIONS OF THE SUPEROXIDE ANION

$O_2^{\cdot -}$ superoxide anion; ${}^1O_2$ singlet oxygen; OH. hydroxyl radical and OH^- hydroxyl anion.

630 nm (Khan and Kaska (1970)).

The established chemical methods used in detection of superoxide anion production during autoxidation of oxygenated cytochrome $P450_{scc}$ were not without significant side reactions and were insufficiently sensitive to allow unequivocal detection of the superoxide radical. Free hydrogen peroxide arising from the dismutation of superoxide anion could not be detected owing to the presence of a peroxidase-like activity in the preparation. The effect of catalase prolonging the presence of hydrogen peroxide decreasing the stability of the oxygenated complex of cytochrome $P450_{scc}$ can only be taken as tentative evidence for the production of hydrogen peroxide during the decay of this complex to the ferric state. These initial experiments may indicate that a more indirect approach to the nature of the iron-oxygen ligand should be made. One possible approach would be the detection of chemiluminescence during the autoxidation of cytochrome $P450_{CAM}$ and suggest that 60% of the electron equivalence can be ascribed to the formation of the superoxide anion. The use of spectroscopic methods similar to those used in the investigation of the structure of oxyhaemoglobin or oxymyoglobin may give information to the nature of the oxygen ligand to cytochrome $P450_{scc}$.

SUMMARY

Cytochrome $P450_{scc}$-cholesterol is a one electron acceptor on titration with NADPH.

Cytochrome $P450_{scc}$-cholesterol can be anaerobically reduced to the ferrous state which on oxygenation forms an oxygenated cytochrome $P450_{scc}$- cholesterol complex. This oxygenated complex autoxidises, in the absence of adrenodoxin, to ferric cytochrome $P450_{scc}$-cholesterol without oxidation of cholesterol. The decay of the oxygenated complex is first order, $k = 9.3 \times 10^{-3} sec^{-1}$ at $4^{o}C$. The rate of autoxidation

is influenced by pH, ionic strength and the nature of bound sterol. The activation energy of autoxidation is 75 kJ $mole^{-1}$.

Investigations into the fate of the electron equivalence as oxygenated cytochrome $P450_{scc}$-cholesterol complex autoxidises to the ferric haemoprotein were initiated and attempts were made to monitor hydrogen peroxide and superoxide anion generation.

CHAPTER 6

PARTIAL RECONSTITUTION OF THE MONOXYGENASE ELECTRON TRANSPORT CHAIN AND THE CATALYTIC TURNOVER OF CYTOCHROME $P450_{SCC}$

Introduction

Cytochrome $P450_{CAM}$ is the terminal oxidase of the electron transport chain associated with hydroxylation of camphor to exo-5-hydroxy-camphor in Pseudomonas putida (Katagiri et al. (1968)). This cytochrome has been purified and crystallised (Yu et al. (1974)) and a reaction cycle postulated (Gunsalus et al. (1972); Estabrook et al. (1972)) for the catalytic turnover of the cytochrome to form a monohydroxylated product.

However the conversion of cholesterol to pregnenolone, catalysed by cytochrome $P450_{scc}$, appears to be a complex series of reactions which may involve stable intermediates. Suggested intermediates are (20S)-20-hydroxycholesterol (Shimizi et al. (1962)); (22R)-22-hydroxycholesterol and (20R 22R)-20, 22-dihydroxycholesterol (Burstein et al. (1975)); cholesterol-(20S)-20-hydroxyperoxide (Van Lierand Smith (1970)) and 20,22-epoxycholesterol (Kraaipoel et al. (1975)).

This chapter reports the nature of the catalytic turnover cycle of cytochrome $P450_{scc}$; the detection of intermediates in the conversion of cholesterol to pregnenolone and the integration of those intermediates into the catalytic cycle.

A variety of monohydroxylated side chain analogues of cholesterol are metabolised to pregnenolone by adrenal mitochondria (Burstein and Gut (1971); Mason et al. (1978a)). Similarly sterols retaining the cholest-5-en-3β-ol nucleus but with variable non-polar side chains are also metabolised to pregnenolone in this system (Arthur et al. (1976)). In the past cholesterol analogues as substrates for the side chain cleavage reaction have been confined to variations in the side chain.

Recently a nuclear di-hydroxylated sterol (7α-hydroxycholesterol) has been shown to form a high spin complex with cytochrome $P450_{scc}$ but has not been established as a substrate for the sterol side chain cleavage reaction (Hume et al. (1979)).

Cytochrome $P450_{scc}$ can be depleted of endogenous cholesterol and this allows readdition of cholesterol of known specific radioactivity: cytochrome $P450_{scc}^{+++}$ + cholesterol $\rightleftharpoons$ cytochrome $P450^{+++}$ - cholesterol.

Cytochrome $P450_{scc}$ is a one electron acceptor and this electron can be introduced from a non-specific chemical donor: cytochrome $P450_{scc}^{+++}$-cholesterol + $1e^-$ → cytochrome $P450_{scc}^{++}$-cholesterol.

Adrenodoxin is a one electron donor (Estabrook et al. (1973)) and is required as donor and effector for the second electron introduced into the oxygenated cytochrome $P450_{scc}$-cholesterol complex. Adrenodoxin can be reduced by non-specific chemical donors (Estabrook et al. (1973)): oxidised adrenodoxin + $1e^-$ → reduced adrenodoxin.

Hence stoicheiometric amounts of cytochrome $P450_{scc}$-cholesterol complex to adrenodoxin and both artificially reduced with one electron under anaerobic conditions should on subsequent oxygenation allow one turnover of the catalytic cycle: reduced adrenodoxin + cytochrome $P450^{++}$-cholesterol + O_2 → H_2O + oxidised adrenodoxin + cytochrome $P450_{scc}^{+++}$-monohydroxylated cholesterol.

RESULTS

Single Cycle Turnover of Cytochrome $P450_{scc}$

Cholesterol, 74.8 nmoles with 0.1 μCi 4-^{14}C cholesterol was added to 2.2 ml low spin cytochrome $P450_{scc}$ (containing 17 nmoles cytochrome $P450_{scc}$/ml) and allowed to incubate 15 hr at 20°C. Adrendoxin, 0.23 ml of stock solution 163 nmoles/ml was added so that the molar ratios of the solution were cholesterol:cytochrome $P450_{scc}$:

adrendoxin:::2:1:1.

The cytochrome $P450_{scc}$-cholesterol complex with adrenodoxin was reduced anaerobically with sodium dithionite added stoicheiometrically to reduce the cytochrome and adrenodoxin. The reduction of cytochrome $P450_{scc}$ was observed by the decrease in absorbance at 390 nm and when constant, the solution was oxygenated by bubbling rapidly with 5 ml oxygen. The solution was extracted with organic solvent and thin layer chromatography performed. A radiochromatogram (Fig. 6.1) of the thin layer plate showed a product (product A) with an R_f value 0.64 compared to cholesterol R_f 0.76 and that of pregnenolone R_f 0.51 in this solvent system. A minor radioactive product (product B) was also noted with an R_f value of 0.39.

Mutliple Cycle Turnovers of Cytochrome P450

If the anaerobic stoicheiometric reduction of cytochrome P450-cholesterol:adrenodoxin complex is repeated for a second time the pattern of products changes (Fig. 6.2). The cycle can be repeated many times and (Fig. 6.3) shows an experiment containing 7 cycles. Expressing the products as a percentage conversion of the total cholesterol added to the incubation, a pattern emerged suggesting that the precursor-product relationship was of the type, cholesterol → product A → product B → pregnenolone.

Precursor-Product Relationship

Product A was extracted from the silica gel of a thin layer plate with chloroform:methanol 2:1 by vol and rechromatographed, reextracted and shown to be a single radioactive species. This sample was added to low spin, substrate depleted cytochrome $P450_{scc}$ reconstituted with adrenodoxin and a single turnover cycle of reduction/oxygenation performed. The resultant thin layer radiochromatogram showed conversion of product A to produce B (Fig. 6.4). Similarly when product B was added to low

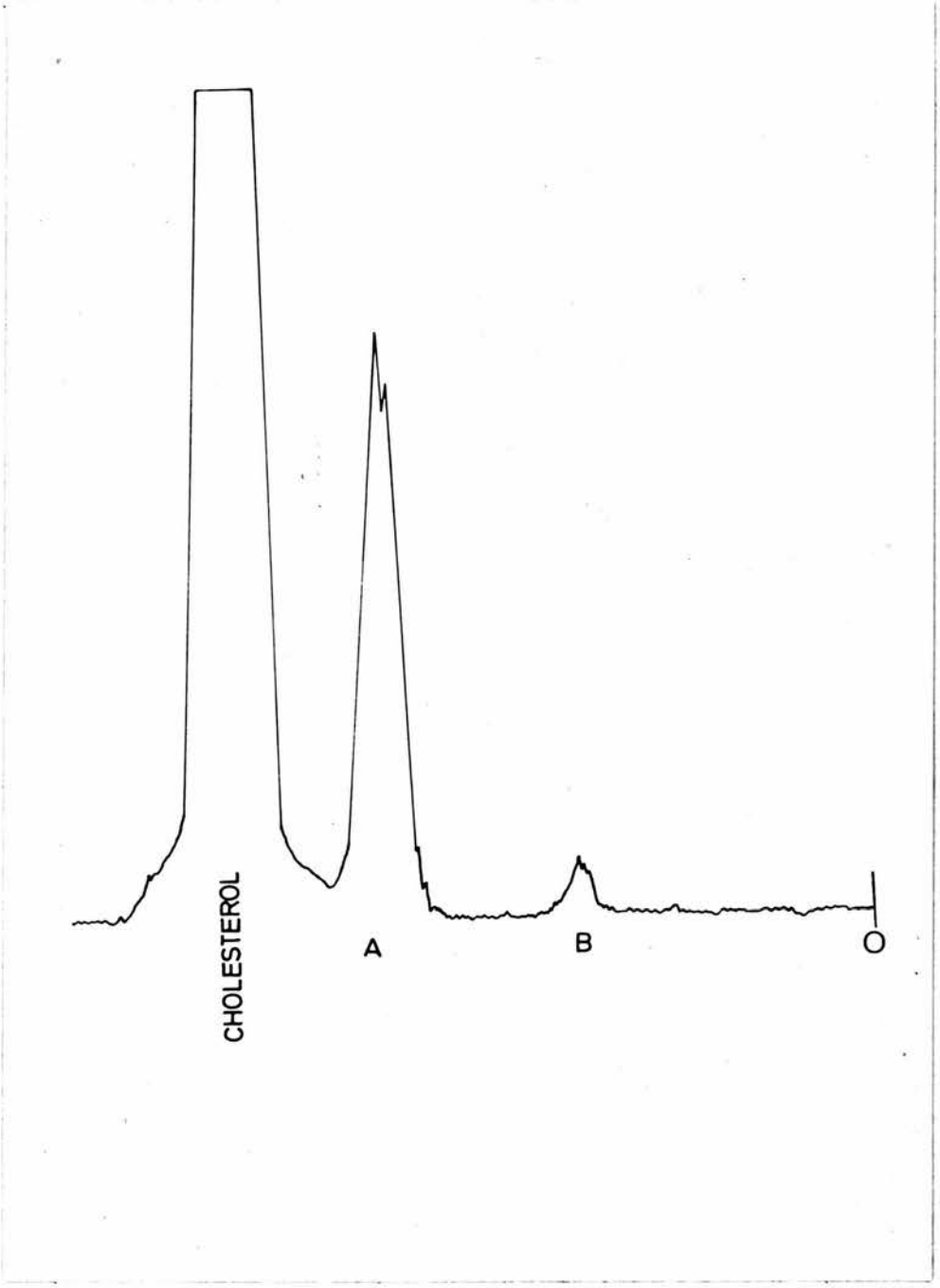


Fig. 6.1. A THIN LAYER RADIOCHROMATOGRAM OF PRODUCTS FORMED AFTER A SINGLE CYCLE OF REDUCTION - OXYGENATION OF (4-^{14}C)-CHOLESTEROL-CYTOCHROME P450$_{scc}$-ADRENODOXIN COMPLEX

Peaks of radioactivity corresponding to cholesterol, product A and product B are shown. Pregnenolone, R_f 0.51 in this system, had a mobility between product A and product B. The cytochrome complex was extracted and chromatography performed as described in Methods. Origin: O

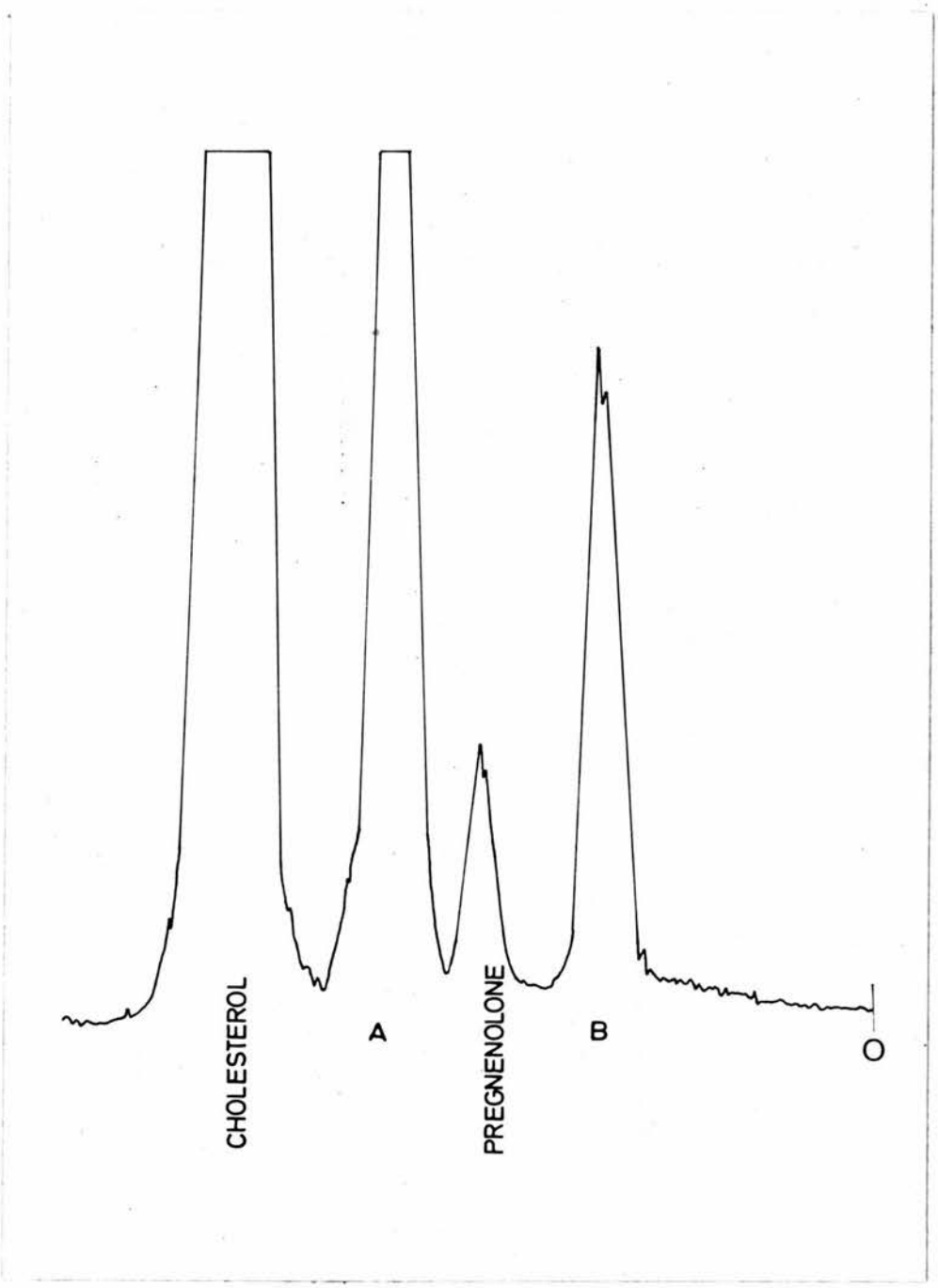


Fig. 6.2. A THIN LAYER RADIOCHROMATOGRAM OF PRODUCTS FORMED AFTER TWO CYCLES OF REDUCTION-OXYGENATION OF (4-^{14}C)-CHOLESTEROL-CYTOCHROME $P450_{scc}$-ADRENODOXIN COMPLEX

Peaks of radioactivity corresponding to cholesterol, product A, product B and pregnenolone are shown. The cytochrome complex was extracted and chromatography performed as described in Methods. Origin: O

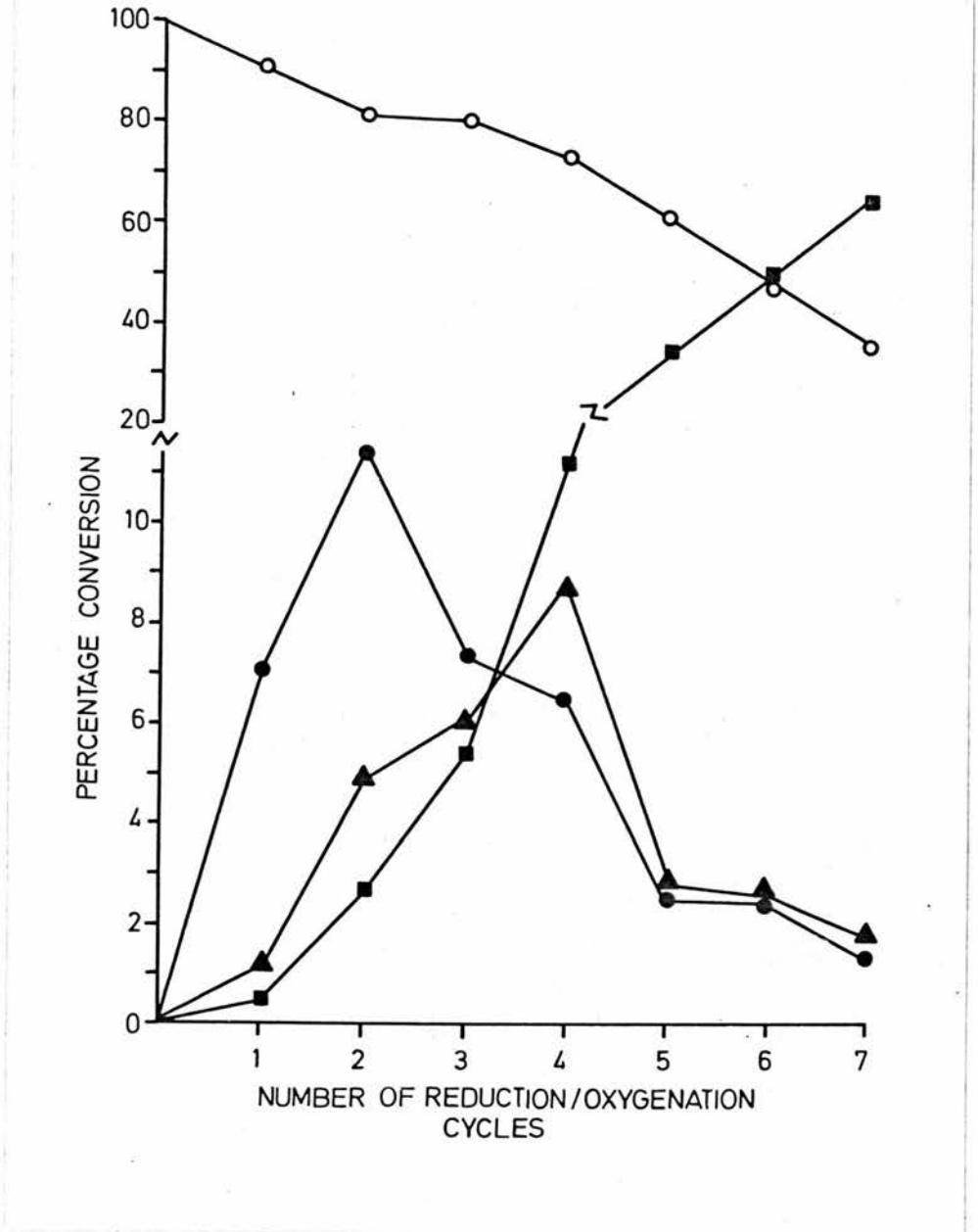


Fig. 6.3. THE PATTERN OF PRODUCT FORMATION DURING SINGLE TURNOVER CYCLES OF ANAEROBIC REDUCTION FOLLOWED BY OXYGENATION OF CYTOCHROME $P450_{scc}$;CHOLESTEROL:ADRENODOXIN COMPLEX

The results are expressed as a percentage conversion of the total (4-^{14}C) cholesterol added to the incubation O —— O cholesterol; ● ——● 22-hydroxycholesterol; ▲ —— ▲ 20,22-dihydroxycholesterol and ■——■ pregnenolone.

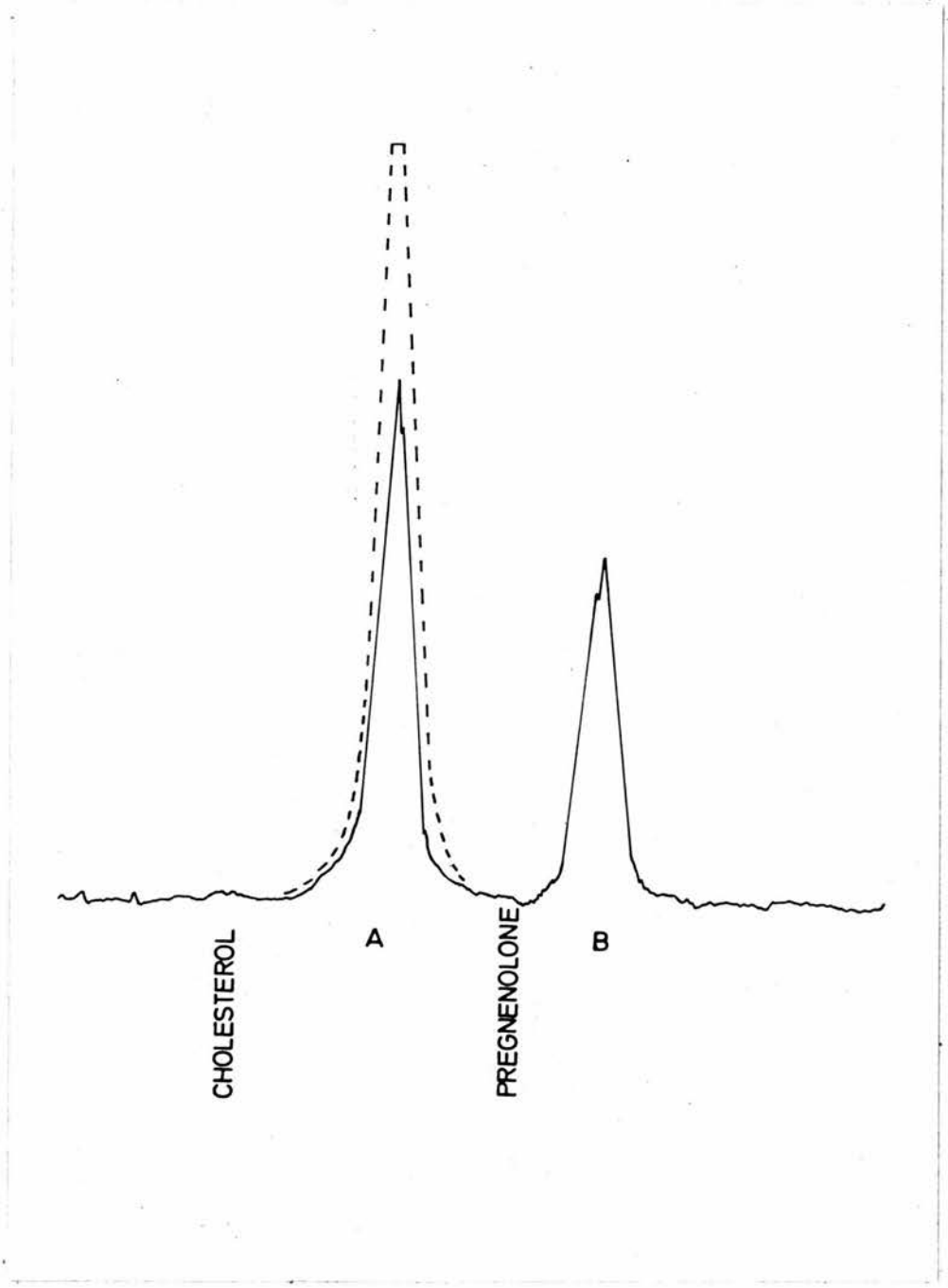


Fig. 6.4. A THIN LAYER RADIOCHROMATOGRAM OF PRODUCTS FORMED AFTER A SINGLE CYCLE OF REDUCTION FOLLOWED BY OXYGENATION OF (4-^{14}C)-PRODUCT A-CYTOCHROME P450-ADRENODOXIN COMPLEX

Peaks of radioactivity of Product A and Product B are shown. ------ represents a radiochromatogram of Product A and ——— a radiochromatogram after a reduction-oxygenation cycle. The cytochrome complex was extracted and chromatography performed as described in Methods.

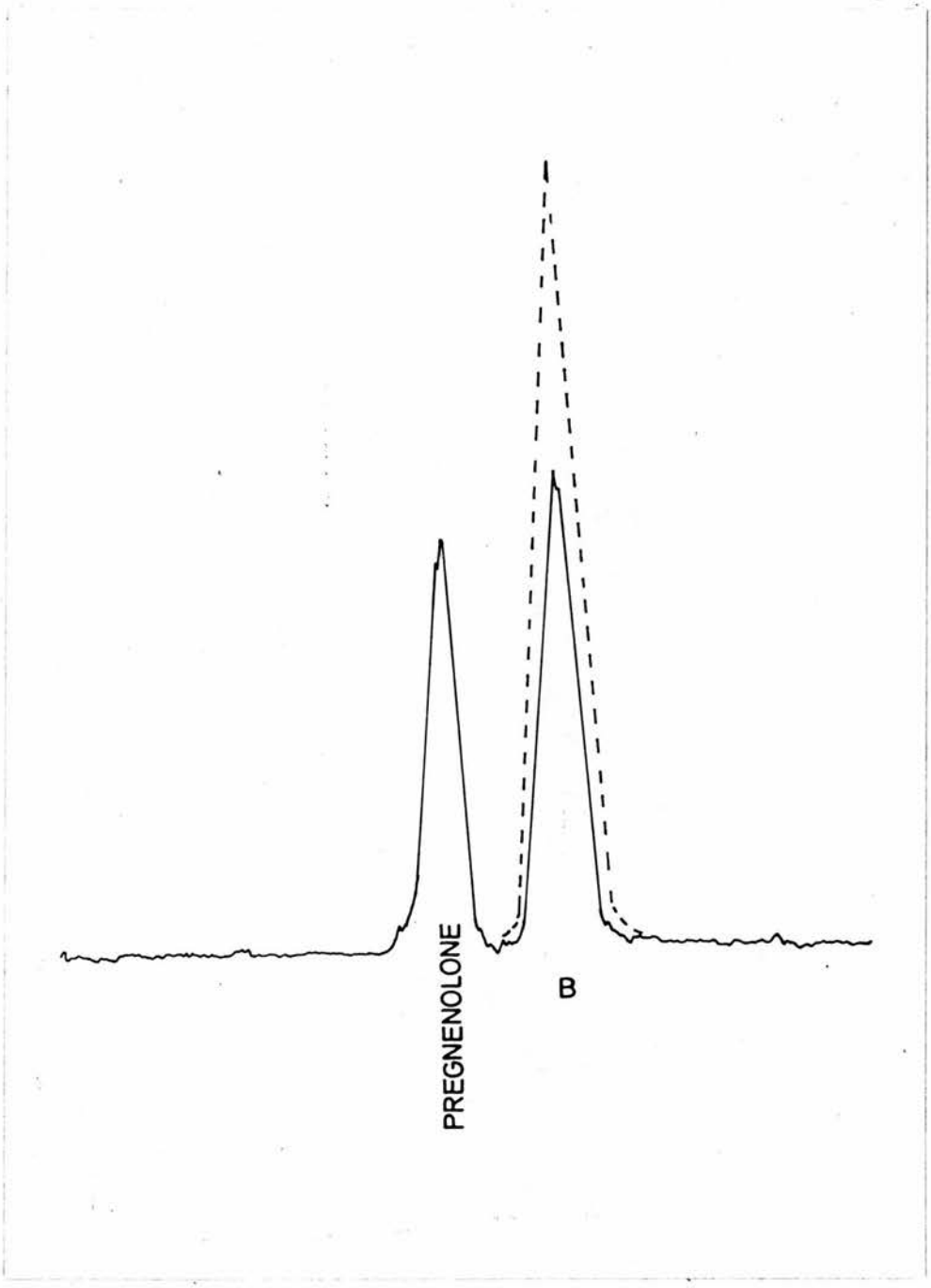


Fig. 6.5. A THIN LAYER RADIOCHROMATOGRAM OF PRODUCTS FORMED AFTER A SINGLE CYCLE OF REDUCTION FOLLOWED BY OXYGENATION OF A ($4-^{14}C$)-PRODUCT B-CYTOCHROME $P450_{scc}$-ADRENODOXIN COMPLEX

Peaks of radioactivity corresponding to Product B and pregnenolone are shown. ------ represents a radiochromatogram of Product B and ——— a radiochromatogram after a reduction-oxygenation cycle. The cytochrome complex was extracted and chromatography performed as described in Methods.

spin substrate depleted cytochrome $P450_{scc}$ and a single cycle turnover performed the product was pregnenolone (Fig. 6.5).

Identification of Intermediates in the Conversion of Cholesterol to Pregnenolone

Thin layer chromatography of products A and B was performed with known standards in the solvent system di-isopropyl ether:petroleum ether:acetic acid, 70:30:2 by volume. The R_f value of product A is similar to the monohydroxylated sterols, (20S)-20-hydroxycholesterol and (22R)-22-hydroxycholesterol. No differentiation in R_f values on TLC could be made between these monohydroxylated sterols. The R_f value of product B was similar to that of 20,22 dihydroxycholesterol (Table 2).

Gas liquid chromatography of non-derivated sterols on a column of 0.5% SE30 on Gas-Chrom Q support as described in the Methods allows separation of (22R)-22-hydroxycholesterol retention time 18.5 min from (20S)-20-hydroxycholesterol, retention time 12.5 min. Product A has a retention time of 18.5 min. Gas liquid chromatography of non-derivatised 20,22-dihydroxycholesterol proved unsatisfactory presumably through dehydration.

The mass spectrum of 22-hydroxycholesterol, (obtained by gas chromatography-mass spectroscopy of the di(trimethylsilyl) ether) isolated after turnover of cytochrome $P450_{scc}$ was identical to the authentic di(trimethylsilyl) derivative (Fig. 6.6) and agreed in detail with the mass spectrum previously described by Burstein et al. (1975). The ion at m/e 173 comprises the fragment C-22 to C-27, elimination of trimethylsilanol from this ion results in the ion m/e 83. The molecular ion was not detected in either standard or isolated material but ions of low abundance were observed at m/e 295 and 343.

TABLE 6.2 R_f values from thin layer chromatography and retention times from gas liquid chromatography of reference and unknown sterols. Thin layer chromatography was performed in diisopropyl ether:petroleum ether:acetic acid 70:30:2 by vol. as described in Methods. Gas liquid chromatography was performed on non-derivatised sterols as described in Methods.

	T.L.C. R_f	G.L.C. (free sterols) Retention Time (mins)
Cholesterol	0.76	10
(20S)-20-Hydroxycholesterol	0.64	12.5
(22R)-22-Hydroxycholesterol	0.64	18.5
(20R,22R)-20,22-Dihydroxycholesterol	0.39	-
Pregnenolone	0.51	-
Product A	0.64	18.5
Product B	0.39	-

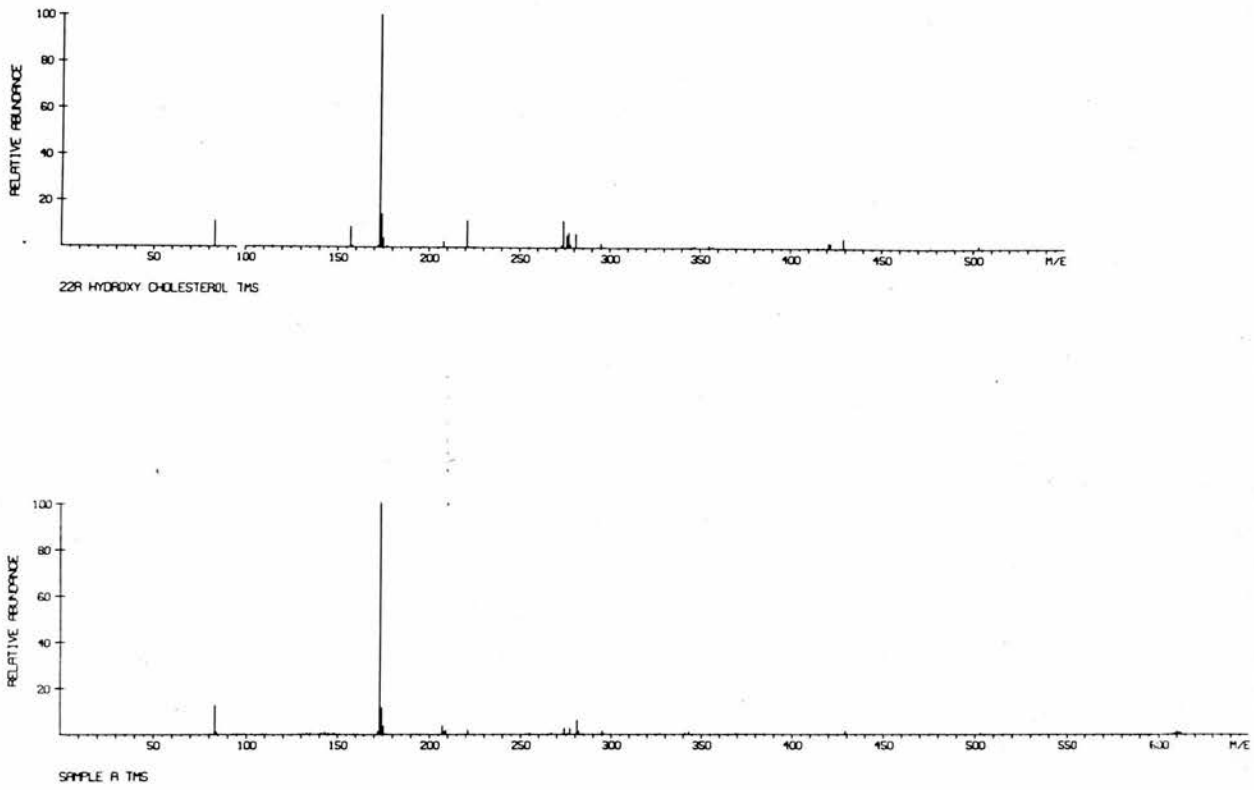


Fig. 6.6. MASS SPECTRA OF 22-HYDROXYCHOLESTEROL

The mass spectrum of 22-hydroxycholesterol (obtained by gas chromatography-mass spectroscopy) of the di(trimethylsilyl)ether) isolated after catalytic turnover of cytochrome $P450_{scc}$ (product A) was identical to the authentic di(trimethylsilyl) derivative and agreed in detail with the mass spectrum previously described by Burstein et al. (1975).

Upper trace: product A.

Lower trace: authentic 22-hydroxycholesterol.

The mass spectrum of 20,22-dihydroxycholesterol (obtained by gas chromatography-mass spectroscopy) of the tri(trimethylsilyl) ether) isolated after turnover of cytochrome $P450_{scc}$ (Fig. 6.7) was identical to the authentic tri(trimethylsilyl) derivative (Fig. 6.7). The molecular ion was not detected despite scanning the peak to give skewed spectra favouring high mass ions. The base peak of the spectrum was m/e 461 (comprising the fragment C-1 to C-21) and other peaks present were m/e 389, 370, 371, 299, 289, 281, 143 and 117.

Efficiency of Single Cycle Turnover of Cytochrome $P450_{scc}$

The efficiency of a single turnover cycle of cytochrome $P450_{scc}$-cholesterol:adrenodoxin can be calculated. The assumption is made that formation of a monohydroxylated sterol from cholesterol requires two electrons; a dihydroxylated product requires four electrons and pregnenolone, six electrons (Shikita and Hall (1973).

For a single turnover of the cycle and with two electrons donated the amount of product formed should be in a 1:1 molar equivalence to cytochrome $P450_{scc}$. The amount of product formed can be calculated from the radioactive conversion knowing the specific radioactivity of added cholesterol and the total amount of cytochrome $P450_{scc}$ present or from absolute determinations of 22-hydroxycholesterol quantitated by gas liquid chromatography. From these calculations the amount of product formed on a single turnover cycle is 30-33% of the molar equivalence of cytochrome $P450_{scc}$. The efficiency of multiple turnover cycles is similar to that of a single cycle.

Products of Autoxidation of Oxygenated Cytochrome P450-Cholesterol

Cholesterol, pre-equilibrated with 4-^{14}C cholesterol, was added in ten-fold molar excess to a 10 μM solution of low spin cytochrome $P450_{scc}$ in 10 mM potassium phosphate buffer pH 7.4 and made anaerobic in a spectrophotometer cuvette. The 4-^{14}C cholesterol was adjusted such

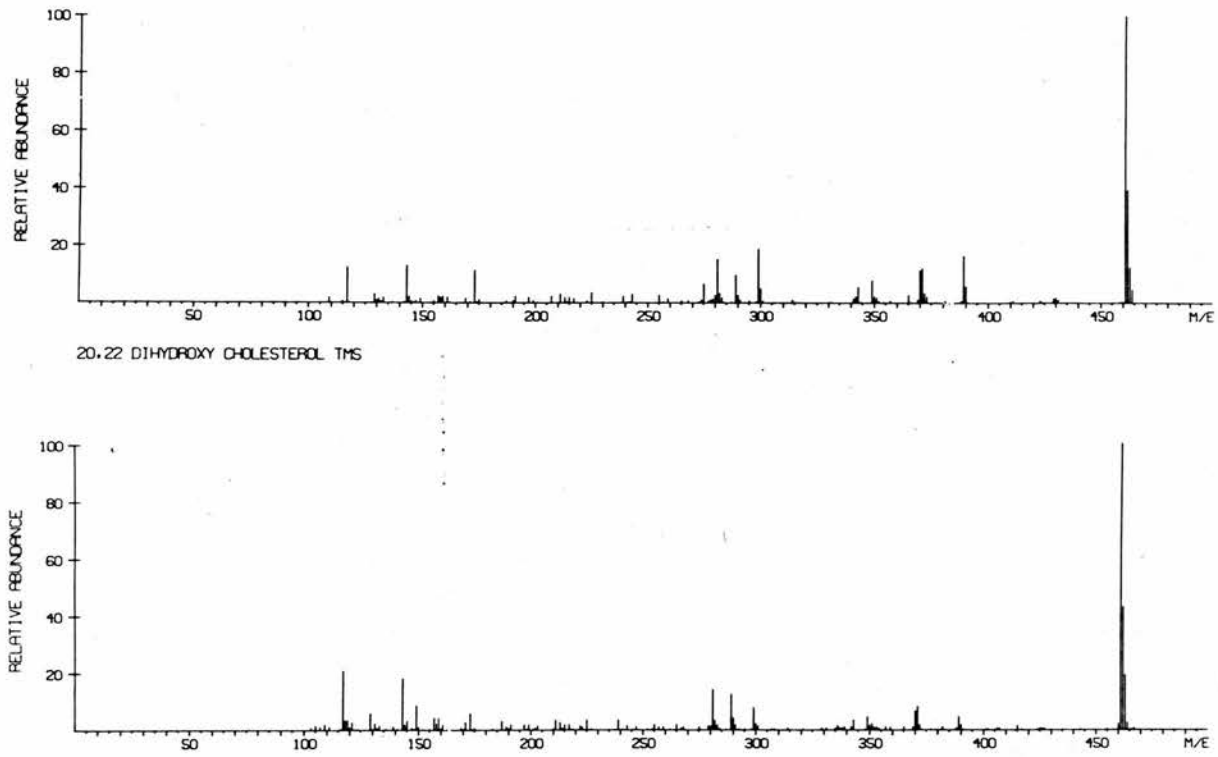


Fig. 6.7. MASS SPECTRA OF 20,22-DIHYDROXYCHOLESTEROL

The mass spectrum of 20,22-dihydroxycholesterol (obtained by gas chromatography-mass spectroscopy) of the tri(trimethylsilyl) ether) isolated after catalytic turnover of cytochrome $P450_{scc}$ (Product B) was identical to the authentic tri(trimethyl)derivative.

Upper trace: product B.

Lower trace: authentic 20,22-dihydroxycholesterol.

that 0.1 μCi was added to an incubation of 2.5 ml. The solution was reduced with stoicheiometric amounts of sodium dithionite, oxygenated, and the reaction followed by absorbance changes at 418 nm until autoxidation was complete. The contents of the cuvette were extracted with organic solvent and thin layer chromatography performed. The developed chromatogram showed no zones of radioactivity other than those associated with (4-^{14}C) cholesterol. This was confirmed by scraping into vials, 1 cm bands of silica gel from the plate, adding scintillation fluid, and determining radioactive counts. No zones of radioactivity were detected other than that associated with (4-^{14}C) cholesterol. The oxygenated cytochrome $P450_{scc}$-cholesterol complex autoxidises to the ferric state without oxidation products of cholesterol being formed.

However, if oxidised adrenodoxin in a molar ratio to cytochrome $P450_{scc}$, was added to the oxygenated cytochrome $P450_{scc}$-cholesterol complex, the product formed was 22-hydroxycholesterol. The efficiency (electrons consumed) in the formation of 22-hydroxycholesterol from cholesterol on addition of oxidised adrenodoxin to oxygenated cytochrome $P450_{scc}$ is around 5% and did not increase by increasing the molar ratio of oxidised adrenodoxin added:oxygenated cytochrome $P450_{scc}$ to 8:1.

Determination of 11β-Hydroxylase Activity of Cytochrome $P450_{scc}$

Deoxycorticosterone, pre-equilibrated with deoxy 1α2α(n)-^{3}H corticosterone, was added to a two-fold molar excess to cytochrome $P450_{scc}$. Adrenodoxin was added in a molar ratio to cytochrome $P450_{scc}$ and the solution reduced anaerobically, then oxygenated. This was repeated for eight cycles. The reaction mixture was extracted with organic solvent and thin layer chromatography performed in chloroform: ethyl acetate, 8:2 by volume. A radiochromatogram of the developed

thin layer plate showed no zones of radioactivity other than that associated with deoxycorticosterone. This was confirmed by scraping into vials, 1 cm bands of silica gel from the plate, adding scintillation fluid and determining radioactive counts.

The Conversion of 7α-Hydroxycholesterol to 7α-Hydroxypregnenolone

(4-^{14}C) 7α-hydroxycholesterol, 160 nmoles with 0.1 μCi, was added to 1.6 mls low spin substrate depleted cytochrome $P450_{scc}$ (80 nmoles) and incubated 15 hrs at 20°C. Adrenodoxin, 80 nmoles, was added to the cytochrome $P450_{scc}$-7α-hydroxycholesterol complex such that the molar ratio of cytochrome $P450_{scc}$:adrenodoxin:7α-hydroxycholesterol was 1:1:2. The adrenodoxin and cytochrome $P450_{scc}$ were reduced stoicheiometrically with sodium dithionite under anaerobic conditions and then oxygenated. This sequence of stoicheiometric anaerobic reduction and oxygenation was repeated for a further eight cycles. A thin layer radiochromatogram of extracted sterols, developed in benzene:ethyl acetate::7:13 by volume showed a single product peak with an R_f 0.3 which corresponded to authentic 7α-hydroxypregnenolone and represented 42% of total radioactivity. The mass spectrum of 7α-hydroxypregnenolone (obtained by gas chromatography-mass spectroscopy of the di(trimethylsilyl)ether) isolated after catalytic turnover of cytochrome $P450_{scc}$ (Fig. 6.8) was indistinguishable from the di(trimethylsilyl) derivatives of authentic 7α-hydroxypregnenolone produced by a photo-oxygenation reaction. The spectra show one intense ion at m/e 386 presumably arising from elimination of trimethyl silanol from the C-7 position. The molecular ion m/e 476 was present in low abundance, 1% of the base ion.

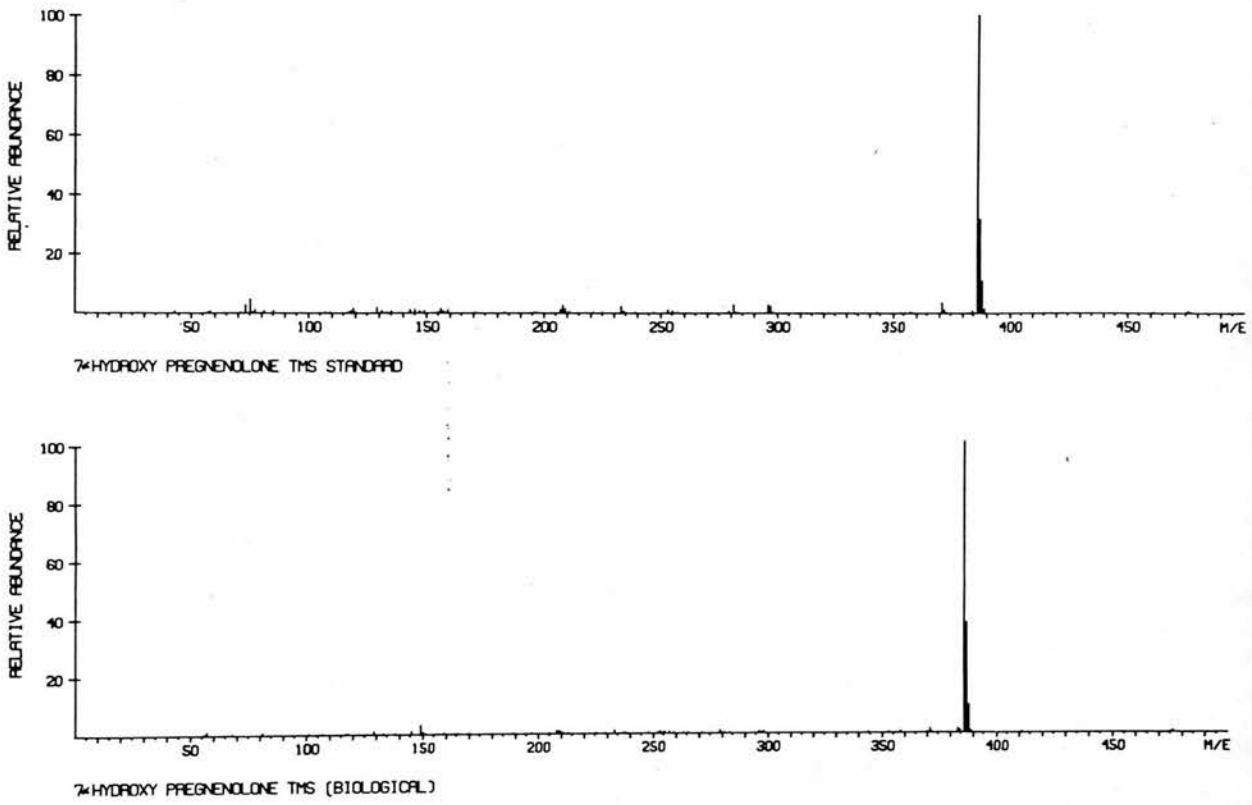


Fig. 6.8. MASS SPECTRA OF 7α-HYDROXYPREGNENOLONE

The mass spectrum of 7α-hydroxypregnenolone (obtained by gas chromatography-mass spectroscopy) of the di(trimethylsilyl) ether) isolated after catalytic turnover of cytochrome $P450_{scc}$ (upper trace) was identical to the authentic di(trimethylsilyl) derivative (lower trace).

DISCUSSION

The detection of intermediates during the side chain cleavage of cholesterol to pregnenolone has been studied. The method was based on the fact that cytochrome $P450_{scc}$ as prepared is depleted of substrate cholesterol; this allows readdition of cholesterol of know specific radioactivity. Cytochrome $P450_{scc}$ is a one electron acceptor and the first electron can be introduced from a non-specific donor such as sodium dithionite. Adrenodoxin is a one electron donor (Estabrook et al. (1973)) and maybe required as an electron donor or as an effector for the second electron introduced into the oxygenated cytochrome $P450_{scc}$-cholesterol complex. Adrenodoxin can be reduced with sodium dithionite (Estabrook et al. (1973)). Hence stoicheiometric amounts of cytochrome $P450_{scc}$-cholesterol complex to adrenodoxin, artifically reduced with one electron, under anaerobic conditions, should on subsequent oxygenation allow one turnover of the cycle.

The evidence presented concerning the reaction sequence of intermediates in the conversion of cholesterol to pregnenolone establishes that the initial oxidative attack catalysed by cytochrome $P450_{scc}$ on cholesterol is at C-22 with the formation of 22-hydroxy-cholesterol followed by oxidative attack at C-20 to form 20,22-dihydroxycholesterol. This sequence agrees with the kinetic studies of Burstein and Gut (1971) on the relative rates of utilisation of (20S)-20-hydroxycholesterol, (22R)-22-hydroxycholesterol and (20R,22R)-20,22-dihydroxycholesterol in an acetone powder preparation of adrenal cortex mitochondria. This evidence excludes the possibility of the initial oxidative attack occurring at C-20 with the formation of 20-hydroxycholesterol as significant in the conversion of cholesterol to pregnenolone under the conditions used in this study.

Cholesterol binding to and subsequent spin state change of cytochrome $P450_{scc}$ appears to be the rate limiting step in the catalytic sequence. The steady state spectrum in intact mitochondria is the low spin cytochrome (Simpson et al. (1971)). This is compared to the catalytic sequence of cytochrome $P450_{CAM}$ where the second electron donation is rate limiting and the steady state spectrum is that of oxygenated cytochrome $P450_{CAM}$ (Tyson et al. (1972)). This difference in rate limiting events has a number of consequences.

Determination of the stoicheiometry of cholesterol side chain cleavage to pregnenolone, in terms of electrons accepted and oxygen consumed, has involved substantial corrections for reactions not associated with hydroxylation (Shikita and Hall (1973)). In this enzyme complex, where substrate binding is rate limiting, low spin substrate depleted cytochrome $P450_{scc}$ could accept electrons with subsequent partial or non-productive cycle turnover producing superoxide or peroxide. The study of the stoicheiometry of single turnover reactions, where the enzyme is primed with substrate, should theoretically circumvent this problem. However the efficiency of a single cycle turnover in a partially reconstituted electron transport chain was low. Adrenodoxin is present in adrenal cortex mitochondria in a 1:1 molar ratio to cytochrome P450, (Estabrook et al. (1973)) although binding studies of purified adrenodoxin to partially purified cytochrome $P450_{scc}$ give dissociation constants of 3 μM for a high affinity binding site (Katagiri et al. (1977)). This suggests that in vitro reconstitution of the electron transport chain may require higher molar ratios of adrenodoxin to cytochrome $P450_{scc}$ for maximal binding of adrenodoxin to cytochrome $P450_{scc}$. The cytochrome $P450_{scc}$ used in the single cycle turnover studies is partially purified and other redox acceptors may be present which reduce the theoretical yield

of hydroxylated product formed from the electron equivalence donated. The possibility also exists of distinct cytochrome P450 s involved in each of the individual steps from cholesterol to pregnenolone. However, no physical separation of these postulated cytochromes has been reported and the ability to form pregnenolone from cholesterol parallels that of pregnenolone formation from (20S)-20-hydroxycholesterol during purification procedures (Shikita and Hall (1973)); (Horie and Watanabe (1975)). Similarly the amount of hydroxylated product formed to electron equivalence donated is similar for single cycles or multiple cycles. In the possible theoretical case of distinct cytochrome P450 s catalysing the individual hydroxylation reactions the efficiency should increase after the first turnover step in the reaction with cholesterol as substrate.

Ferric cytochrome $P450_{scc}$-cholesterol when reduced stoicheiometrically with sodium dithionite under anaerobic conditions and then bubbled with oxygen to form the oxygenated cytochrome $P450_{scc}$-cholesterol complex, failed to form oxidation products of cholesterol during the subsequent autoxidation of this complex. However, when oxidised adrenodoxin was added to this oxygenated cytochrome $P450_{scc}$-cholesterol complex, 22-hydroxycholesterol was formed. The efficiency of the mixed function oxidase in terms of electrons required to effect conversion of cholesterol to product under the specified conditions described, appears to be about five per cent. This is a lower figure than that obtained in experiments on pre-equilibration and stoicheiometric reduction of adrenodoxin and cytochrome $P450_{scc}$-cholesterol prior to oxygenation and raises the question of the importance of the association kinetics of cytochrome $P450_{scc}$ and adrenodoxin and whether this is different with oxidised or reduced adrenodoxin. The role of adrenodoxin at the stage of electron donation to the oxygenated

cytochrome $P450_{scc}$-cholesterol complex cannot be answered by the experiments described in this chapter. The possibilities are that adrenodoxin acts solely as an electron donor or as an effector or has both functions. In the first case where oxidised adrenodoxin is added to the oxygenated cytochrome $P450_{scc}$-cholesterol complex, one would have to postulate that some of the adrenodoxin be reduced by reversal of electron flow from a ferrous cytochrome $P450_{scc}$ molecule; this reduced adrenodoxin in turn donating an electron to an oxygenated cytochrome $P450_{scc}$-cholesterol complex. In similar experiments when oxidised putidaredoxin was added to oxygenated cytochrome $P450_{CAM}$-camphor complex reversal of electron flow through the iron sulphur protein was postulated (Lipscomb et al. (1976)). However, one could argue that oxidised adrenodoxin may not be reduced and that intermolecular transfer of electrons takes place between cytochrome $P450_{scc}$ molecules. The observation that relatively simple dithiols such as dihydrolipoic acid added in 10^3-10^6 molar excess to oxygenated cytochrome $P450_{CAM}$-camphor will allow hydroxylation of camphor in the absence of putidaredoxin (Lipscomb et al. (1976)), may indicate that iron sulphur proteins may have a dual role at this stage of the cycle. In essence, we can confirm the essential requirement of adrenodoxin for the overall reaction of cholesterol side chain cleavage as observed by Kimura et al. (1973) and suggest that the step of electron donation to oxygenated cytochrome $P450_{scc}$-cholesterol may be the site for this requirement.

The failure to detect 11β-hydroxylation of deoxycorticosterone under the conditions described or to observe a Type I spectral change on addition of deoxycorticosterone to cholesterol depleted cytochrome $P450_{scc}$ suggests a complete separation of the cytochrome P450 associated with the side chain cleavage of cholesterol from that associated with

11β-hydroxylation. The concept of distinct cytochrome P450 s catalysing side chain cleavage of cholesterol and 11β-hydroxylation of deoxycorticosterone has been confirmed by Ramseyer and Harding (1973), Shikita and Hall (1973) and Katagiri et al. (1975). Corticosterone, a substrate for the 18-hydroxylation reaction present in adrenal cortex mitochondria, when added to the cholesterol depleted cytochrome $P450_{scc}$ preparation failed to induce a Type I spectral change. The 18-hydroxylase activity in intact mitochondria is low compared to that of side chain cleavage of cholesterol or 11β-hydroxylation of deoxycorticosterone. It has been suggested that 18- and 11β-hydroxylations are controlled by the same gene locus and that these activities are supported by a single enzyme. During the preparation of cytochrome $P450_{scc}$ no enzymatic assays of 18-hydroxylase activity were made.

The structural requirements for the sterol side chain cleavage reaction has been investigated with sterols retaining the cholest-5-en-3β-ol nucleus but with variable non-polar side chain (Arthur et al. (1976)). Similarly a variety of monohydroxylated side chain analogues are metabolised to pregnenolone by adrenal mitochondria (Burstein and Gut (1971); Mason et al. (1978a)). Cholesterol analogues for the side chain cleavage reaction have been confined to variations in the side chain. The evidence presented establishes that a nuclear monohydroxylated sterol, 7α-hydroxycholesterol, is metabolised to 7α-hydroxypregnenolone. The 7α-hydroxycholesterol induces a low to high spin state transition of substrate depleted cytochrome $P450_{scc}$, binding more rapidly and to a greater extent than the natural substrate cholesterol or the side chain monohydroxylated sterols.

SUMMARY

Addition of equimolar amounts of adrenodoxin to cytochrome $P450_{scc}$-cholesterol, followed by stoicheiometric reduction under anaerobic conditions and then oxygenation allows single catalytic turnover cycles of cytochrome $P450_{scc}$ to be studied. This has allowed detection of intermediates in the conversion of cholesterol to pregnenolone and a precursor-product sequence of cholesterol - 22-hydroxycholesterol - 20,22-dihydroxycholesterol-pregnenolone to be established.

Addition of oxidised adrenodoxin to oxygenated cytochrome $P450_{scc}$-cholesterol results in formation of 22-hydroxycholesterol; in the absence of added adrenodoxin no oxygenated product of cholesterol is formed.

Multiple turnover cycles with deoxycorticosterone as substrate for cholesterol depleted cytochrome $P450_{scc}$ failed to show deoxycorticosterone to corticosterone.

Multiple turnover cycles with 7α-hydroxycholesterol as substrate for cholesterol depleted cytochrome $P450_{scc}$ confirmed that the side chain of this nuclear monohydroxylated cholesterol is cleaved to form 7α-hydroxypregnenolone.

CHAPTER 7

DISCUSSION

The most commonly postulated pathway for conversion of cholesterol to pregnenolone entails a consecutive series of reactions - cholesterol-(20S)-20-hydroxycholesterol-(20R,22R)-20,22-dihydroxycholesterol-pregnenolone (Shimizi et al. (1961); Constantopoulos and Tchen (1961); Shimizi et al. (1962)). This scheme was postulated on the basis that (i) cholesterol is cleaved between carbons 20 and 22 giving rise to a six carbon fragment (Staple et al. (1956); Constantopoulos et al. (1962)). (ii) (20S)-20-hydroxycholesterol is formed from(4-^{14}C) cholesterol by bovine adrenal cortex homogenates in the presence of trapping agents (Solomon et al. (1956)) and (iii) (20S)-20-hydroxy-cholesterol and (20R,22R)-20,22-dihydroxycholesterol are more efficiently transformed to pregnenolone than is cholesterol (Shimizi et al. (1961); Shimizi et al. (1962)). Constantopoulos et al. (1962) described the accumulation of (20R,22R)-20,22-dihydroxycholesterol with a bovine adrenocortical preparation in the presence of pregnenolone. However Koritz and Hall (1964); Hall and Koritz (1964) and Simpson and Boyd (1967) were unable to observe the formation of these sterols from cholesterol and explained this by the suggestion that the cleavage of the cholesterol side chain is a concerted reaction in which the intermediates do not appear in the medium and that only the final product, pregnenolone, is released when the reaction is completed.

The possible involvement of 20-hydroxy, 22-ketocholesterol as an intermediate has been suggested (Shimizi et al. (1962)), convertible to pregnenolone but at a slower rate than (20R,22R)-20,22-dihydroxy-cholesterol. However experiments by Constantopoulos et al. (1962,1966) have effectively eliminated the formation of a 22-keto group in the conversion of (20S)-20-hydroxycholesterol to pregnenolone.

Chaudhuri et al. (1962) showed that (22R)-22-hydroxycholesterol was effectively converted to pregnenolone. Burstein, Gut and colleagues (1969,1970) have exploited a kinetic approach to the study of intermediates in the side chain cleavage of cholesterol using radioactive sterols in an acetone powder preparation of adrenal mitochondria. These authors conclude that the slowest step in the formation of pregnenolone is the conversion of cholesterol to the monohydroxylated product, that the formation of (20S)-20-hydroxycholesterol from cholesterol is slower than that of (22R)-22-hydroxycholesterol and the rate of (22R)-22-hydroxycholesterol utilisation to pregnenolone is greater than that of (20S)-20-hydroxycholesterol. However most of the pregnenolone formed does not appear via any of these hydroxylated products and the authors assume a concerted attack of oxygen to form (20R,22R)-20,22-dihydroxycholesterol which is oxidatively cleaved to pregnenolone. Similar pathways involving a direct conversion of (20S)-20-hydroxycholesterol and (22R)-22-hydroxycholesterol to pregnenolone, not mediated via the dihydroxycholesterol, have also been suggested.

Van Lier and Smith (1970) have shown that cholesterol-(20S)-20-hydroperoxide is converted to (20R,20R)-20,22-dihydroxycholesterol by rearrangement of the peroxide, a mechanism not requiring reducing equivalents or oxygen. If this is a significant mechanism then a single molecule of oxygen should donate atoms to both positions. However the evidence of Burstein et al. (1976) shows the distribution of the oxygen atoms in 20,22-dihydroxycholesterol after incubation of cholesterol with $^{18}O_2$ and $^{16}O_2$ to be consistent with introduction from two different oxygen molecules. A subsequent publication by Van Lier et al. (1976) proposes that the mechanism of side chain cleavage of cholesterol involves three consecutive <u>in situ</u> oxidations

in a manner consistent with those of Burstein et al. (1970).

Kraaipoel et al. (1975) have suggested an alternative mechanism for side chain cleavage of cholesterol. Cholesterol is dehydrogenated to form cholesten-5,20-diene-3β-ol, subsequent oxygenation forming 20,22-epoxycholesterol which is hydrated to (20R,22R)-20,22-dihydroxy-cholesterol with an oxygen dependent cleavage of the dihydroxycholesterol to pregnenolone. The incorporation of (20S)-20-hydroxycholesterol or (22R)-22-hydroxycholesterol into this pathway is by dehydration at the C20-22 bond. Another possible route, not suggested by the authors, to form hydroxylated cholesterol would be through isomerisation of 20,22-epoxycholesterol as is well documented for arene oxides (Jerina and Daly (1974)).

The formation of an epoxide during a similar dealkylation to the sterol side chain cleavage reaction has been established in the conversion of sitosterol to cholesterol in the silkworm with the intermediate formation of fucosterol 24,28 oxide (Morisaki et al. (1972) and Awata et al. (1975)). The mammalian liver microsomal cytochrome P450 dependent systems have been shown, with aromatic compounds as substrates, to form phenols and diols through intermediate arene oxides (Jerina and Daly (1974)), although in certain cases direct formation of a phenol can occur without an intermediate epoxide (Tomaszewski et al. (1975)).

Burstein et al. (1976) have shown that (22R)-(22-^{18}O) 22-hydroxy-cholesterol and (20S)-(20-^{18}O) 20-hydroxycholesterol are converted to the respective dihydroxycholesterols and that the formed glycols and substrates at the end of incubations have the same ^{18}O abundance as the starting material. The evidence presented by Burstein et al. (1976) showing the distribution of oxygen atoms in 20,22-dihydroxycholesterol after sequential incubation with $^{18}O_2$ and $^{16}O_2$ is consistent with the

introduction of two atoms of oxygen into the diol from two different oxygen molecules. If 20,22-epoxycholesterol were an intermediate then one of the oxygen atoms in 20,22-dihydroxycholesterol would be derived from molecular oxygen and the other from water. The four stereoisomers of 20,22-epoxycholesterol were synthesised chemically by Morisaki et al. (1976) and incubated with partially purified cytochrome $P450_{scc}$ in the presence of an appropriate electron supply. None of these derivatives was significantly converted to pregnenolone although a slight inhibition of side chain cleavage of radioactive cholesterol by these epoxycholesterols was noted but there occurred no trapping of the radioactivity by these compounds. However, Burstein et al. (1976) have shown, using an acetone powder preparation of adrenal mitochondria, that (20R,22S)-20,22-epoxycholesterol yielded one fifth the amount of pregnenolone compared to a similar incubation with cholesterol but (20R,22R)-20,22-epoxycholesterol was not metabolised. Burstein et al. (1976) and Morisaki et al. (1976) have shown that no significant (20R,22R)-20,22-dihydroxycholesterol is formed on incubation of (E)- or (Z)-20(22)-dehydrocholesterol.

Luttrell et al. (1972) and Hochberg et al. (1974) consider that the hydroxycholesterol compounds to be by-products of the side chain cleavage of cholesterol and that the true intermediates are short lived reactive complexes of oxygenated steroidal species and the metalloenzyme. The authors present evidence that (20R)-20-t-butyl-5-pregnene-3β,20-diol and (20R)-20-(p-tolyl)-5-pregnene-3β, 20 diol are converted to pregnenolone by bovine adrenocortical mitochondria and as position C22 is fully substituted this site is unavailable for hydroxylation. The authors suggest an ionic or radical reaction for the basic mechanism of side chain cleavage of cholesterol. However Morisaki et al. (1976) incubating (20S)-20-tolypregn-5-en-3β,20 diol

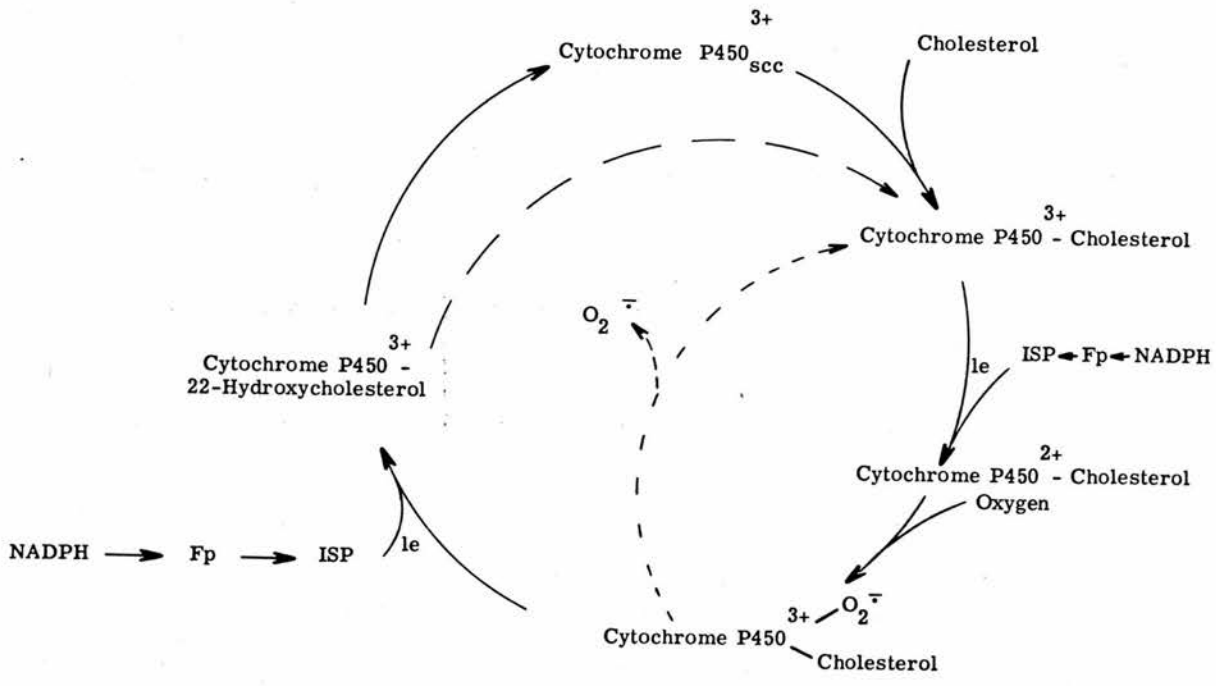


Fig. 7.1. PROPOSED CATALYTIC SEQUENCE FOR THE CHOLESTEROL SIDE CHAIN CLEAVAGE REACTION

A single turnover cycle of cytochrome $P450_{scc}$ is shown with cholesterol as substrate and the formation of the first stable intermediate 22-hydroxycholesterol which is retained at the active site, a further catalytic cycle produces 20,22-dihydroxycholesterol. The oxygenated complex of cytochrome $P450_{scc}$-cholesterol is shown in only one of the possible resonance forms. ISP: iron sulphur protein (adrenodoxin) and Fp: NADPH adrenodoxin reductase.

with partially purified cytochrome $P450_{scc}$ failed to show significant conversion to pregnenolone.

The evidence presented concerning the reaction sequence of intermediates in the conversion of cholesterol to pregnenolone establishes that the initial oxidative attack catalysed by cytochrome $P450_{scc}$ on cholesterol is at C-22 with the formation of 22-hydroxycholesterol followed by oxidative attack at C-20 to form 20,22-dihydroxycholesterol. This sequence agrees with the kinetic studies of Burstein and Gut (1971) on the relative rates of utilisation of (20S)-20-hydroxycholesterol (22R)-22-hydroxycholesterol and (22R,22R)-20,22-dihydroxycholesterol in an acetone powder preparation of adrenal cortex mitochondria. This evidence excludes the initial oxidative attack occurring at C-20 and hence the formation of 20-hydroxycholesterol is significant in the conversion of cholesterol to pregnenolone. Similarly if cholesterol (20S)-20-hydroperoxide were an intermediate (Van Lier and Smith (1970)), rearrangement of the peroxide might result in 20,22-dihydroxycholesterol as the initial product isolated. The sequence established excludes 20,22-epoxycholesterol as an intermediate in the catalytic cycle (Kraaipoel et al. (1975)) as the second product is a glycol 20,22-dihydroxycholesterol rather than epoxide. The evidence presented cannot exclude the speculation that the true intermediates in the conversion of cholesterol to pregnenolone might be a short lived, reactive complex of oxygenated steroidal species and a metalloenzyme (Hochberg et al. (1974); Luttrell et al. (1972)). Certainly short lived, unstable intermediates of this type must exist at the stage of second electron donation to the oxygenated cytochrome $P450_{scc}$-sterol complex whether this sterol be cholesterol, 22-hydroxycholesterol or 20,22-dihydroxycholesterol.

A catalytic sequence for the cholesterol side chain cleavage reaction is proposed (Fig. 7.1). Cholesterol binds to the ferric enzyme and induces a spin change to a high spin state; the ferric-cholesterol complex accepts one electron to form a ferrous-cholesterol complex which in turn interacts with molecular oxygen to form a ternary complex termed oxygenated cytochrome P_{450scc}- cholesterol. The oxygenated form of cytochrome $P450_{scc}$-cholesterol accepts a second electron to form as yet an ill-defined, irreversible intermediate whose decay results in the formation of 22-hydroxycholesterol. However, instead of product release and regeneration of the low spin substrate depleted cytochrome $P450_{CAM}$ (Gunsalus et al. (1972)), 22-hydroxycholesterol is retained and a further catalytic cycle produces 20, 22-dihydroxycholesterol. Similarly, 20, 22-dihydroxycholesterol is retained at the active site of cytochrome $P450_{scc}$ and a further cycle results in oxidative cleavage to pregnenolone and presumably isocaproic aldehyde.

The failure to detect significant conversion of the isomers of 20,22-epoxycholesterol and 20(22)-dehydrocholesterol to pregnenolone (Burstein et al. (1976); Morisaki et al. (1976) and the evidence presented in this thesis would seem substantial evidence for sequential oxygenation of cholesterol at C-22 then C-20 to form 20,22-dihydroxycholesterol rather than the hydration of the epoxide, 20,22-epoxycholesterol (Kraaipoel et al. (1975)). This could be further substantiated by experiments not as yet attempted on the cholesterol side chain cleavage, namely:

a) the demonstration of the absence of an NIH shift during the formation of 22-hydroxycholesterol, and

b) the demonstration of a significant isotope effect in the formation of 22-hydroxycholesterol from cholesterol or in the formation of 20,22-dihydroxycholesterol from cholesterol.

The migration and retention of substituents that occur during the monoxygenase catalysed formation of phenols from most aromatic substrates has been termed the 'NIH shift' (Guroff et al. (1967)). The occurrence of this shift suggested that the 'aromatic hydroxylations' are actually epoxidations and that the intermediate arene oxides undergo a ready isomerisation to phenols with a concomitant migration and retention of substituents (Jerina and Daly (1974)). The formation of an arene oxide from a deuterated aromatic substrate should proceed without a significant isotope effect since carbon-hydrogen bonds are not broken in the oxidation (Guroff and Daly (1967); Daly and Jerina (1967)).

When oxidation at a saturated carbon is considered either the substrate must be activated by loss of hydride, hydrogen atom, or a proton to form a readily oxidised carbonium ion, radical, or carbanion (abstraction), or transfer of the equivalent of singlet oxygen to the substrate must occur. Enzymatic hydroxylation at saturated carbon often shows a primary isotope effect and invariably proceeds with a high degree of retention of configuration (Daly (1971)). These mechanisms are compatible with an insertion mechanism involving transfer of a single oxygen atom. Abstraction pathways, while generally less likely for energetic reasons, are equally compatible with primary isotope effects (Tomaszewski et al. (1975)). In general aromatic hydroxylations are considered to have arene oxides as intermediates, show no significant isotope effects but have an NIH shift (Jerina and Daly (1974)). However, examples of aryl hydroxylation are known with significant isotope effect (Tanabe et al. (1970); Tomaszewski et al. (1975)). In general aliphatic hydroxylations show significant isotope effects (Oesch et al. (1971)) but again exceptions to this generalisation are known (Ullrich (1968); Tagg et al. (1967)).

In 1964, Hamilton remarked on the similarity of many of the monoxygenase catalysed hydroxylations to the carbene and nitrene reactions and suggested that monoxygenases catalyse their reactions by an oxygen transfer or oxenoid mechanism. In no case has the structure of an actual enzyme oxenoid reagent been positively identified but speculation has been made on the oxenoid reaction as the mechanism of cytochrome P450 dependent reactions (Hamilton et al. (1974)). It is generally agreed that the oxidation of substrate apparently occurs when an electron is transferred to a Fe^{++} form of the enzyme that has substrate and oxygen bound to it. Thus, at least formally, there would appear to be three possibilities for the oxenoid reagent:

a) an enzyme - Fe^{+} - molecular oxygen complex,

b) an enzyme - Fe^{+++} - peroxide compound, and

c) an enzyme - Fe^{+++++} - oxygen atom complex.

The Fe^{+} enzyme would presumably be a resonance hybrid of Fe^{+}-porphyrin, Fe^{++}-reduced porphyrin (by one electron), and Fe^{+++}-reduced porphyrin (by two electrons). Similarly, the formal Fe^{+++++} intermediate would have resonance contributions involving Fe^{++++} and Fe^{+++}, with oxidised porphyrin structures (Hamilton (1969)). The first possibility appears unlikely as the oxygen would have an excess of electrons rather than being electron deficient, a condition necessary for model compound reactions (Hamilton (1974)). The second alternative has been discounted on the basis that Fe^{+++}-peroxide complexes do not react readily with alkanes or aromatic compounds (Hamilton (1974)). The third alternative is regarded as a possible structure for the oxenoid reagent (Hamilton (1974); van Lier and Rousseau (1976)). Van Lier and Rousseau (1976) have proposed a reaction mechanism (Fig. 7.2) for the cholesterol side chain cleavage reaction with a ferryl ion complex, Fe^{++}-oxygen atom as the reactive species not only for the

sequential hydroxylations at C_{22} and C_{20} but also for the oxidative cleavage of the C_{20}-C_{22} bond.

The reaction involving the cleavage of the C_{20}-C_{22} carbon bond of 20,22-dihydroxycholesterol to pregnenolone and a six carbon fragment has a number of unusual features. This reaction appears to require molecular oxygen and a source of reducing equivalents in the form of NADPH (Fiet et al. (1971)). The cleavage of 20,22-dihydroxycholesterol to pregnenolone is inhibited by carbon monoxide and the photochemical action spectrum on reversal of this carbon monoxide inhibition has a peak at 451 nm suggesting that cytochrome P450 is involved in the reaction (Hall et al. (1975)). The stoicheiometry of conversion of 20,22-dihydroxycholesterol to pregnenolone has been investigated by Shikita and Hall (1974) and the authors conclude the reaction has a ratio of pregnenolone produced:NADPH consumed:oxygen consumed of 1:1:1. However these measurements did involve substantial corrections for undefined background reactions not, according to the authors, involved in the production of pregnenolone.

The nature of the six carbon fragment formed during cholesterol side chain cleavage to pregnenolone was initially considered to be isocaproic acid (Lynn et al. (1954); Staple et al. (1956)). The six carbon fragment was shown to be formed in a 1:1 molar ratio to pregnenolone (Constantopoulos and Tchen (1961)) but the authors considered that the initial product formed was isocaproic aldehyde and not isocaproic acid. Burstein and Gut (1971) observing the formation of isocaproic aldehyde, isohexyl alcohol and isocaproic acid during the cholesterol side chain cleavage reaction in human, chicken and bovine adrenal material made a number of observations.

The authors observed that in individual adrenal preparations from the same species isocaproic aldehyde was the major product formed although the ratio to isocaproic acid and isohexyl alcohol varied. Comparison of species showed that chicken and human adrenal produced isohexyl alcohol as the major product. Incubation of isocaproic aldehyde with bovine adrenals led to the formation of isohexyl alcohol and isocaproic acid while a similar incubation with human tissue led to the formation of isohexyl alcohol. The authors conclude that isocaproic aldehyde is the primary product of the cholesterol side chain cleavage reaction and that it is followed by reduction to isohexyl alcohol and oxidation to isocaproic acid.

Takemoto et al. (1968) have presented evidence to show that in the presence of $^{18}O_2$ no oxygen is incorporated into either pregnenolone or isocaproic aldehyde on incubation of 20,22-dihydroxycholesterol with a bovine adrenal preparation. No evidence can be presented for the fate of the oxygen molecule, apparently required in stoicheiometric amounts to the substrate. If the molecular oxygen is incorporated into water then two moles of water would be formed per mole of pregnenolone formed. A conclusion of this nature would not be consistent with this reaction being termed a mixed function oxidation according to the definition of Mason (1957).

A reaction mechanism for the cholesterol side chain cleavage reaction has been proposed by Van Lier and Rousseau (1976) based on the ferryl ion-oxygen complex catalysing three *in situ* oxidations at the C_{22}-H, C_{20}-H and C_{20}-$_{22}$ bonds to give pregnenolone and isocaproic aldehyde as the final products (Fig. 7.2a). The authors postulate formation of the ferryl-oxygen atom complex by protonation of a ferrous-superoxide form with the intermediate formation of the hydroperoxy state, elimination of the hydroxyl ion results in cleavage of the O-O bond. The sequential formation of 22-hydroxy-

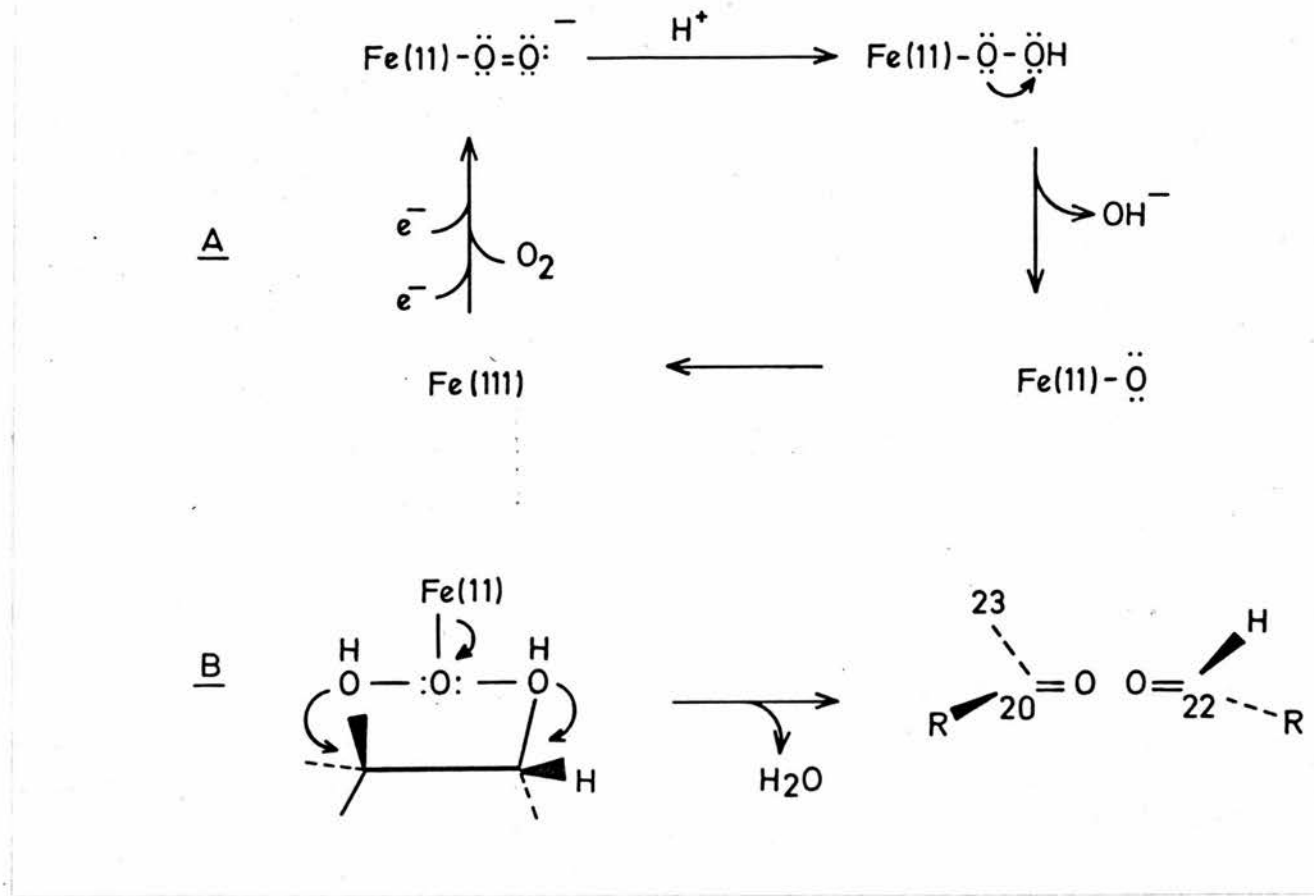


Fig. 7.2. REACTION MECHANISM FOR CHOLESTEROL SIDE CHAIN CLEAVAGE PROPOSED BY VAN LIER AND ROUSSEAU (1976).

The authors postulate formation of the ferryl-oxygen atom complex by protonation of a ferrous-superoxide form with the intermediate formation of the hydroperoxy state, elimination of the hydroxyl ion results in cleavage of the O-O bond (Fig. 7.2a). The cleavage of the C_{20}-$_{22}$ bond of 20,22-dihydroxycholesterol is envisaged as a two electron abstraction by the ferryl ion complex resulting in the formation of pregnenolone and isocaproic aldehyde (Fig. 7.2b).

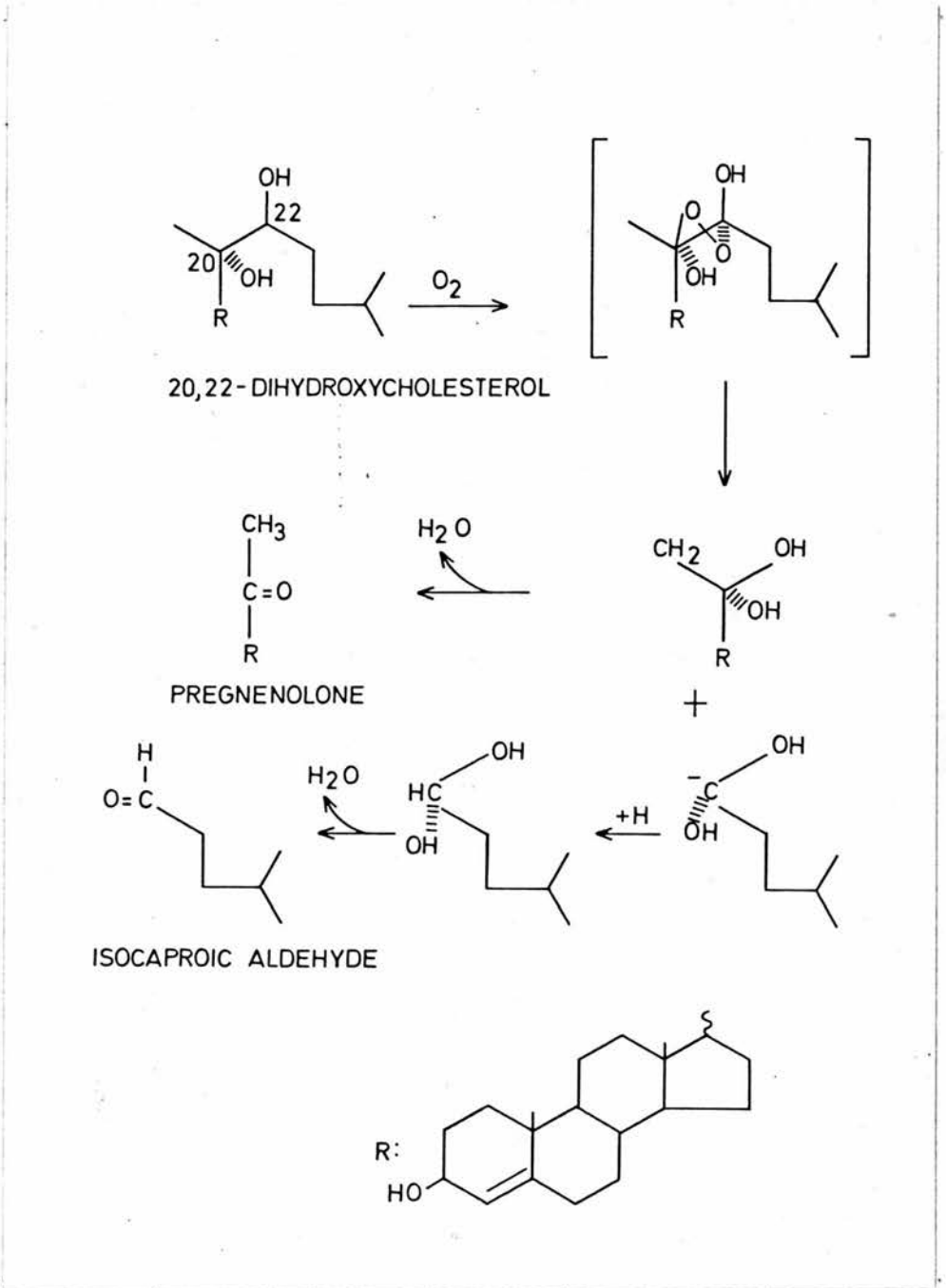


Fig. 7.3. PROPOSED MECHANISM FOR THE CLEAVAGE OF THE C_{20}-$_{22}$ BOND OF 20,22-DIHYDROXYCHOLESTEROL

In this model both atoms of a molecule of oxygen are incorporated into 20,22-dihydroxycholesterol forming a peroxy bridge across the C_{20}-$_{22}$ bond and facilitating cleavage of the bond to yield isocaproic alcohol and 3β,20,20-trihydroxypregnene-5-ene. Elimination of a molecule of water from each of these products would yield isocaproic aldehyde and pregnenolone.

cholesterol and 20,22-dihydroxycholesterol by either an initial abstraction of hydrogen or a direct insertion of oxygen over the C-H bond would be consistent with the evidence presented in this thesis and the evidence of Burstein and Gut (1971); Morisaki et al. (1976). Van Lier and Rousseau (1976) envisage the cleavage of the C_{20}-C_{22} bond of 20,22-dihydroxycholesterol by a two electron abstraction by the ferryl ion complex resulting in the formation of pregnenolone and isocaproic aldehyde (Fig. 7.2b). This final stage in the cholesterol side chain cleavage reaction envisages cytochrome $P450_{scc}$ as an oxidase with no oxygenase function and with the elimination of two moles of water per mole of product formed.

In considering potential mechanisms for the cleavage of the C_{20}-C_{22} bond of 20,22-dihydroxycholesterol, Van Lier and Rousseau (1976) have speculated on an oxidase function for cytochrome $P450_{scc}$ a major divergence from the traditional view of cytochrome $P450_{scc}$ as a monoxygenase. In a similar speculative vein one could propose that cytochrome $P450_{scc}$ might act as a dioxygenase at the stage of cleavage of the C_{20}-C_{22} bond of 20,22-dihydroxycholesterol. This would involve incorporation of both atoms of a molecule of oxygen into 20,22-dihydroxycholesterol forming a peroxy bridge across the C_{20}-C_{22} bond and facilitating 3β,20,22-trihydroxypregnene-5-ene formation (Fig. 7.3). Elimination of a molecule of water from each of these products would yield isocaproic aldehyde and pregnenolone. The oxygen atom eliminated in water from each of the initial products would have to be derived from that oxygen incorporated to form the peroxy bridge across the C_{20}-C_{22} bond to satisfy the lack of incorporation of oxygen during the cleavage of 20,22-dihydroxycholesterol. Mechanisms similar to this reaction scheme have been proposed for

C-C bond cleavage by dioxygenase systems such as 3,4-dihydroxyphenyl-acetate dioxygenase and for cathecol 1,2 - and 2,3 - dioxygenase (Dorn and Knackmuss (1978)).

In conclusion elucidation of the nature of the C_{20}-C_{22} bond cleavage of 20,22-dihydroxycholesterol may reveal a reaction mechanism singular to cytochrome P450 dependent reactions.

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